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REVIEW ARTICLE

The use of mode of action information in risk assessment: Quantitative key events/dose-response framework for modeling the dose-response for key events

Ted W. Simon[1], S. Stoney Simons Jr.[2], R. Julian Preston[3], Alan R. Boobis[4], Samuel M. Cohen[5], Nancy G. Doerrer[6], Penelope A. Fenner-Crisp[7], Tami S. McMullin[8], Charlene A. McQueen[9], J. Craig Rowlands[10], and RISK21 Dose-Response Subteam[11]

[1]Ted Simon LLC, Winston, GA, USA, [2]Steroid Hormones Section, NIDDK/LERB, National Institutes of Health, Bethesda, MD, USA, [3]U.S. EPA/National Health and Environmental Effects Research Laboratory, Research Triangle Park, NC, USA, [4]Imperial College London, London, UK, [5]University of Nebraska Medical Center, Omaha, NE, USA, [6]ILSI Health and Environmental Sciences Institute, Washington, DC, USA, [7]Consultant, North Garden, VA, USA, [8]Dow Corning, Midland, MI, USA, [9]U.S. EPA/National Health and Environmental Effects Research Laboratory, Research Triangle Park, NC, USA, and [10]The Dow Chemical Company, Toxicology and Environmental Research & Consulting, Midland, MI, USA, [11]ILSI Health and Environmental Sciences Institute, Washington, DC, USA.

Abstract

The HESI RISK21 project formed the Dose-Response/Mode-of-Action Subteam to develop strategies for using all available data (in vitro, in vivo, and in silico) to advance the next-generation of chemical risk assessments. A goal of the Subteam is to enhance the existing Mode of Action/Human Relevance Framework and Key Events/Dose Response Framework (KEDRF) to make the best use of quantitative dose-response and timing information for Key Events (KEs). The resulting Quantitative Key Events/Dose-Response Framework (Q-KEDRF) provides a structured quantitative approach for systematic examination of the dose-response and timing of KEs resulting from a dose of a bioactive agent that causes a potential adverse outcome. Two concepts are described as aids to increasing the understanding of mode of action—Associative Events and Modulating Factors. These concepts are illustrated in two case studies; 1) cholinesterase inhibition by the pesticide chlorpyrifos, which illustrates the necessity of considering quantitative dose-response information when assessing the effect of a Modulating Factor, that is, enzyme polymorphisms in humans, and 2) estrogen-induced uterotrophic responses in rodents, which demonstrate how quantitative dose-response modeling for KE, the understanding of temporal relationships between KEs and a counterfactual examination of hypothesized KEs can determine whether they are Associative Events or true KEs.

Abbreviations: AChE acetyl cholinesterase, AE associative event, AOP adverse outcome pathway, As3mt arsenic methyltransferase, AUC area under the curve, BMDL benchmark dose lower confidence limit, BMR benchmark response, BPA bisphenol A, BrdU bromodeoxyuridine, BuChE butyrylcholinesterase, ChE cholinesterase, CPF chlorpyrifos, CYP450 cytochrome P450, DES diethylstilbestrol, DMA[III] dimethylarsinic acid (reactive metabolite trivalent), DMA[V] dimethylarsinic acid, DMPS dimercaptopropanesulfonic acid, DR dose-response, EC European Commission, EC_{50} median effective concentration, EFSA European Food Safety Authority, EPA Environmental Protection Agency (US), ER estrogen receptor, ERα estrogen receptor alpha, HESI Health and Environmental Sciences Institute, HRF Human Relevance Framework, ILSI International Life Sciences Institute, IVIVE in vitro to in vivo extrapolation, KE key event, KEDRF Key Events/Dose-Response Framework, L-NAME L-NG-nitroarginine methyl ester, ModF modulating factor, MIE molecular initiating event, MOA mode of action, MOE margin of exposure, NOAEL no-observed-adverse-effect level, NRC National Research Council, OECD Organisation for Economic Co-operation and Development, OP organophosphate, PBPK physiologically based pharmacokinetic, PD pharmacodynamic, PON1 Paraoxonase 1, PR progesterone receptors, Q-KEDRF Quantitative Key Events/Dose-Response Framework, QSAR quantitative structure-activity relationship, RBC red blood cell, REACH Registration, Evaluation, Authorisation and Restriction of Chemicals, RIP140 receptor interacting protein 140, SAM S-adenosyl methionine, SRC-1 steroid receptor coactivator-1, TCPy 3,5,6-trichloro-2-pyridinol, TDV traditional dose value, WoE weight of evidence.

Address for correspondence: Nancy G. Doerrer, ILSI Health and Environmental Sciences Institute, 1156 15th Street, NW, Washington, DC 20005, USA. Tel: +202-659-3306. E-mail: ndoerrer@hesiglobal. org; URL: http://www.risk21.org

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Introduction

As society progresses through the second decade of the 21st century, there is increased pressure to embrace new ideas and new information in the practice of toxicology and risk assessment. Modern biological science has provided many assessment tools—genomics, transcriptomics, proteomics, metabolomics, and others—that enable scientists to dissect and ultimately understand the biological pathways underlying toxicity. Disruption of these pathways is associated with adverse outcomes.

The progression of this understanding of these adverse outcome pathways fosters and enables the use of these new tools in the practice of chemical risk assessment (Ankley et al. 2010, NRC 2007). What is needed is the knowledge of the biological pathways that underlie a given toxicity and an estimate of the degree or amount of disruption each pathway can tolerate without the occurrence of pathway-specific toxicity (Boekelheide and Andersen 2010, Boekelheide and Campion 2010, Hartung and McBride 2011). The use of mode of action (MOA) currently is the most reliable way for developing sufficient knowledge and understanding of these biological pathways.

RISK21 project

For a number of years, the International Life Sciences Institute (ILSI) Research Foundation has assembled cross-disciplinary working groups to examine current risk assessment approaches for evaluating dose-response and identifying safe exposure levels (Julien et al. 2009). Recently, these efforts were applied to four categories of bioactive agents—food allergens, nutrients, pathogenic microorganisms, and environmental chemicals—and from the lessons learned, a common analytical framework was developed for understanding MOA—the Key Events/Dose-Response Framework (KEDRF; Boobis

et al. 2009, Buchanan et al. 2009, Julien et al. 2009, Ross et al. 2009, Taylor et al. 2009).

The present paper describes ways to incorporate information about the timing of occurrence and quantitative dose-response of Key Events (KE) into the KEDRF. This expanded framework is known as the Quantitative Key Events/Dose-Response Framework or Q-KEDRF. In one sense, this is a "how-to" paper, which describes methods to incorporate additional information for understanding the particulars of the MOA of a chemical. In addition to a discussion of these methods, examples are provided for illustration.

Dose-response/Mode-of-Action Subteam

A central issue in 21st century toxicology and risk assessment is dose-response analysis and its extrapolation to human exposure levels. Building on the KEDRF, the Dose-Response (DR)/Mode-of-Action (MOA) Subteam within the ILSI Health and Environmental Sciences Institute's (HESI's) RISK21 project was formed to develop a clear strategy for using all available data (*in vitro, in vivo,* and *in silico*) in both qualitative and quantitative ways to develop the methods to be used in next-generation risk assessments of substances. The gathering of these various types of data is best accomplished in a tiered fashion suggested by the red triangle labeled as "Toxicity" in the upper left portion of Figure 1.

The DR/MOA Subteam has three main objectives: 1) to provide a forum to discuss approaches to dose extrapolation in human health risk assessment; 2) to address how an understanding of MOA will influence low-dose extrapolation; and 3) to enhance the existing MOA/Human Relevance Framework (HRF) and KEDRF. Specifically, this third objective aims to use quantitative dose-response and temporal information about both KEs and the adverse outcome in a more robust way. Consistent with all HESI projects, participation in the Risk21
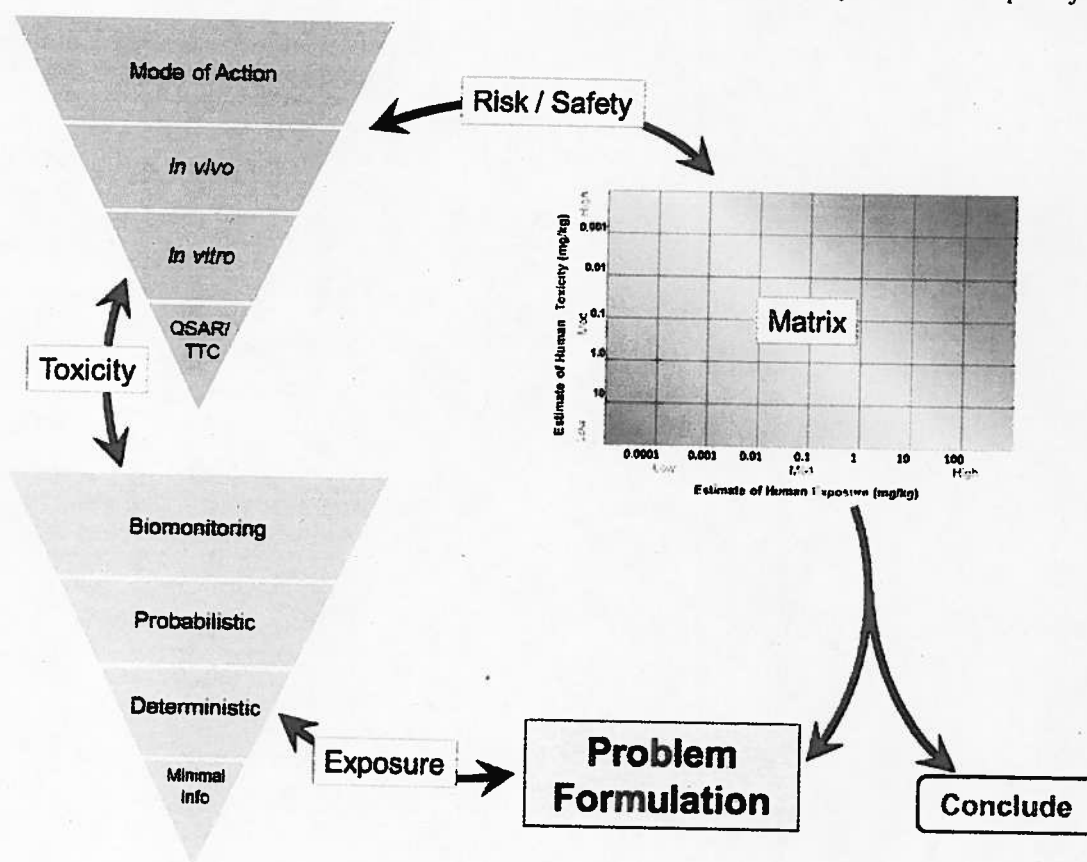
Figure 1. The HESI RISK21 Roadmap and Matrix.

Dose-Response Subteam included tripartite representation from government, academia, and industry, with subteam co-leadership provided by expert scientists from academia and industry.

History and uses of MOA/HRF frameworks

MOA is defined specifically in the US Environmental Protection Agency's (EPA's) 2005 *Guidelines for Carcinogen Risk Assessment* as follows:

> ... *a sequence of Key Events and processes, starting with interaction of an agent with a cell, proceeding through operational and anatomical changes, and resulting in cancer formation. A "key event" is an empirically observable precursor step that is itself a necessary element of the mode of action or is a biologically based marker for such an element. Mode of action is contrasted with "mechanism of action," which implies a more detailed understanding and description of events* (USEPA 2005a).

While necessary, single KEs by themselves are not usually sufficient for the adverse outcome to occur, as noted by Julien et al. (2009):

> *Hence, a key event is a necessary, though not a sufficient, step in a process that results in a specific adverse effect.*

Julien et al. (2009) also provide some historical perspective on the concept of MOA and broadened the definition as the "fundamental biological events and processes that underlie the effect of a bioactive agent". In risk assessment, consideration of MOA likely originated from the work of Lehman-McKeeman et al. (1989) on male rat nephrotoxicity associated with accumulation of alpha 2μ-globulin, the work of Cohen and Ell-wein (1990) and Cohen (1995) on bladder carcinogenesis, and that of Faustman et al. (1997) on the evaluation of mechanisms of developmental toxicity.

The KEDRF provides a structured approach for systematic examination of KEs that occur between the initial dose of a bioactive agent and the final or apical effect of concern (Julien et al. 2009). Here, not only are the timing of KEs and the quantitative aspects of dose-response examined, but also two additional concepts for understanding MOA are discussed—Associative Events (AEs) and Modulating Factors (ModFs). These concepts were defined in Andersen et al. (2014). AEs essentially provide biomarkers for KEs, and a full definition is provided in a later section. ModFs affect the timing and/or dose-response of KEs and include variability in homeostasis or repair capacities, adaptive or immune mechanisms, enzyme polymorphisms, and other biological factors. The nature and strength of ModFs varies between individuals and in the same individual over time. Life stage, disease state, genetics, life-style, and other factors underlie this inter- and intra-individual variability. The Q-KEDRF provides a means to incorporate ModFs in specific situations (described below), and thus, to understand how these result in distributions of popula-tion sensitivity in the dose-response of the various KEs and, ultimately, the adverse outcome.

MOA included in regulatory guidance

Government regulatory agencies around the world have incorporated MOA/HRFs into guidance documents because of their ability to inform risk assessments. For example, the European Commission (EC) has incorporated MOA in its risk assessment guidance for industrial chemicals and biocides, and the US EPA's *Guidelines for Carcinogen Risk Assessment* specifically emphasizes the use of MOA information for interpreting and quantifying the potential cancer risks to humans (EC-JRC 2003, USEPA 2005a). In addition, EPA's *Supplemental Guidance for Assessing Susceptibility from Early-Life Exposure to Carcinogens* (or Supplemental Guidance) also relies on knowledge of the MOA (USEPA 2005b). The EPA has also drafted a *Framework for Determining a Mutagenic Mode of Action for Carcinogenicity* that is also based upon MOA, but this guidance has not yet been finalized (USEPA 2007). Health Canada considers MOA in development of drinking water guidelines and pesticide resistance management labeling (Health Canada 1999, 2009, 2011, Liteplo and Meek 2003).

The European Food Safety Authority (EFSA) includes a MOA assessment in its guidance on Harmonizing Cancer and Non-cancer Risk Assessment Approaches (EFSA 2005). MOA is recommended in the EC Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH) Regulation guidance for conducting a chemical safety assessment, and in the new "classification, labelling, and packaging" regulation on chemical substances and mixtures (EC 2008). The Organisation for Economic Co-operation and Development (OECD) recommends using MOA to support the building of chemical categories or when using read-across approaches (http://www.oecd.org/chemicalsafety/risk-assessment/groupingofchemicalschemicalcategoriesandread-across.htm). OECD has further embraced the concept of MOA in its recent use of adverse outcome pathways (AOPs; Ankley et al. 2010, OECD 2013). With the push to use more systematic and weight-of-evidence (WoE) approaches in risk assessment, both the recognition of the value and importance of the MOA/HRF and KEDRF and their use in risk assessments will increase.

MOA reduces uncertainty and informs quantitative risk assessment

MOA is a fundamental component of risk assessment for the classification of carcinogens and systemic toxicants, and informing the choice of whether a nonlinear or linear approach to low-dose extrapolation is appropriate. Evaluators can use quantitative kinetic and/or dynamic data considered in MOA analysis in at least five ways. These are listed below, along with specific examples:

1) replace default species extrapolation factors;
2) evaluate more directly the relevant concentrations in the target tissue;
3) determine the most representative dose metric;
4) choose the most appropriate quantitative dose-response model; and
5) assess quantitatively the overall relevance to humans.

Replacement of the default toxicodynamic component of the species extrapolation factor was based on species-dependent differences in the dose-response for AHR activation between

humans and rodents in a risk assessment for dioxin based on the 2006 NTP cancer bioassay (Budinsky et al. 2014, NTP 2006, Simon et al. 2009). The understanding gained by investigation into the MOA of small intestinal carcinogenesis by hexavalent chromium led to the identification of the flux of hexavalent chromium entering each segment of the small intestine as the best measure of concentration affecting the target tissue (Kirman et al. 2012, Thompson et al. 2014). The extensive work on the MOA of the pesticide chlorpyrifos (discussed in detail below) enabled the recent identification of brain cholinesterase inhibition as the most appropriate dose metric for a risk assessment based on cholinesterase inhibition (Reiss et al. 2012). An examination of the MOA of acrylamide-induced mammary tumors in F344 rats suggested that nonlinear low-dose extrapolation was a more appropriate method than linear extrapolation (Maier et al. 2012). Last, the Q-KEDRF is part of the MOA/human relevance framework (MOA/HRF) and the purpose of this larger framework is the assessment of human relevance (Boobis et al. 2006, Boobis et al. 2008, Cohen et al. 2003, Cohen et al. 2004, Cohen and Arnold 2011, Meek et al. 2003, Meek 2008, Seed et al. 2005, Meek et al. 2014a, Meek et al. 2014b).

An understanding of MOA is also needed to account for the role of metabolism in various tissues and to decide which early metabolic changes may be KEs. This understanding enables the evaluator to account for induction or inhibition of metabolism of a particular chemical and for potential first-pass effects that may increase or decrease toxicity due to metabolite formation or reduction in the systemic dose of the parent compound. Variations in patterns of toxicity with different metabolic profiles exist across species, strains and sexes in animals and across potentially susceptible subgroups and different life stages in humans. These variations need to be considered so that appropriate and defensible quantitative adjustments can be made for purposes of incorporation of these differences into risk assessments. The overall result is that MOA information can reduce uncertainties in risk assessments in a number of areas.

MOA is the foundation of 21st century toxicology testing and risk assessment

The interpretation of traditional animal toxicity studies for their relevance to humans is difficult, at times impossible, and, more often than not, fraught with controversy (Seok et al. 2013, Beyer et al. 2011, Gori 2013, NRC 1983). These studies generally use high doses resulting in considerable uncertainty when attempting to extrapolate the effects observed in animals to humans, especially when humans are experiencing much lower environmental exposures (NRC 1983). Aspects of this interpretation no less important than human relevance include: 1) the advances in understanding MOA, including the molecular and cellular events responsible for toxicity; 2) the desire to refine, reduce and replace the use of animals in regulatory toxicity testing; and 3) the need for toxicity evaluations for the large number of chemicals in commercial use. In response to these issues, the National Research Council (NRC) developed recommendations on toxicity testing that incorporated new *in vitro* and *in silico* technologies and computational systems biology to complement, and eventually replace, whole animal testing. The new strategy was presented in a report titled

Toxicity Testing in the 21st Century: a Vision and a Strategy (NRC 2007).

The report emphasized the importance of relating events leading to toxicity in the context of perturbations in biologic functions, some of which may be reversible or may represent biologically appropriate adaptations to stressors. Twenty–first century risk assessment uses the knowledge of MOA to link together perturbations in biological pathways observed in humans, in animals, in experiments with *in vitro* systems, and even those predicted by quantitative structure–activity relationships (QSAR) or other computational methods with the goal of determining the likelihood of adverse health outcomes in humans (upper left box in Figure 2).

One vital aspect of this new strategy and the vision of 21st century risk assessment is the development of appropriate prediction models (Adeleye et al. 2014, Judson et al. 2014, Patlewicz et al. 2013). Statistical approaches that attempt to correlate high throughput assay results with adverse outcomes appear to possess a level of predictivity no better than that derived from chemical structure (Thomas et al. 2012). The realization of this difficulty has fostered the curation of AOPs for use in prediction models (Landesmann et al. 2013, OECD 2013, Vinken 2013). In addition, attempts are being made to develop broad categories of MOAs for the purpose of exploiting extant knowledge across categories in a new application of read across (Briggs et al. 2012, Thomas et al. 2013, Vink et al. 2010). Understanding MOA seems to be a necessary part of eventual use of AOPs for risk assessment. Both dose and time contribute to the development of a biologically adverse response—hence, knowledge of MOA requires a detailed understanding of the dose- and time-dependency of the steps that lead from the initial interaction with a chemical to a specific toxic effect (Rowlands et al. 2014).

The Q-KEDRF—a tool for understanding MOA

MOA provides a link between exposure and the risk of adverse health outcomes—but only when the observed pathway perturbations can be characterized in terms of KEs. An important aspect of the definition of a KE is that its occurrence is necessary for the apical event. The other part of the definition is that a KE is "empirically observable." Necessity, as part of the definition, allows one to develop a counterfactual experiment for a putative KE (Figure 2, Box B2) and actually pose the question of whether it truly is a KE—if the event does not occur, will the adverse outcome occur?

Organizing questions and a toolbox for the Q-KEDRF

Box 1 provides a set of organizing questions for MOA as a prelude to applying the Q-KEDRF for specific MOA analyses. These general questions were developed from the charge questions provided to three expert panels in a workshop held at NIEHS to evaluate nuclear receptor-mediated MOAs for liver carcinogenicity (Budinsky et al. 2014, Corton et al. 2014, Andersen et al. 2014, Elcombe et al. 2014). The questions are sorted into three general areas, but in practice, there will likely be considerable overlap between the questions. Attempting to answer these questions will provide anyone engaged in MOA analysis with an understanding of the extent of knowledge.

Box 2 provides three overall categories of schemes for concise organization of the MOA information resulting from tackling the questions in Box 1. Examples of these methods are given from the papers resulting from the nuclear receptor workshop (Budinsky et al. 2014, Corton et al. 2014, Andersen et al. 2014, Elcombe et al. 2014). Necessarily, the graphical techniques, save for the flow chart, will be quantitative. Although not mentioned specifically
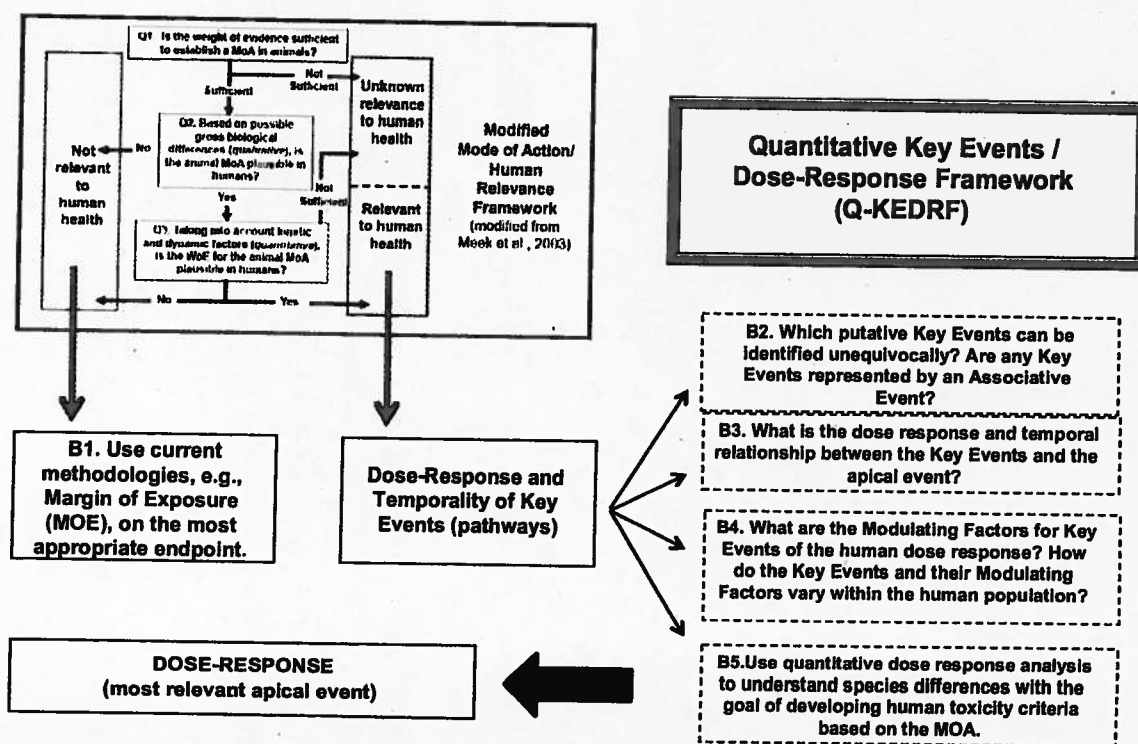

Figure 2. Quantitative Key Events/Dose-Response Framework (Q-KEDRF) and Its Relationship to the Mode of Action/Human Relevance Framework.

Box 1. Organizing questions for mode of action analysis.

Organizing Questions for MOA Consideration
- What is the proposed MOA to be evaluated by the IPCS Human Relevance Framework and modified Bradford Hill considerations?
- Which events are necessary and thus truly key events (KEs)?
- Which events are associative events (AEs)?
- What are the modulating factors (ModFs)?
- Is the proposed MOA likely to be relevant to humans?

Organizing Questions for Quantitative Dose-Response Consideration
- Are extant data sufficient for establishing dose-response relationships for proposed KEs?
- Are extant data sufficient for dose-response modeling of proposed KEs?
- What are the data gaps?
- Does the current understanding support a threshold or non-threshold DR and low dose extrapolation approach?
- On either theoretical or practical grounds, is there a dose or area-under-the-curve (AUC) level insufficient for one or more KEs or the adverse outcome (AO) to occur?

Organizing Questions for Using MOA in Risk Assessment
- Does the weight-of-evidence suggest an appropriate model or approach for the dose-response assessment?
- If so, what are the key data gaps?
- Using a value-of-information (VOI) approach (NRC, 2009; Meek et al., 2014a, b), what data would have the highest value?

in Box 2, exposure–response arrays used in the Toxicological Profiles from the Agency for Toxic Substances and Disease Registry (ATSDR) and the newer Toxicological Reviews from EPA's IRIS program could be organized around proposed KEs within one or more hypothesized MOAs.

WoE considerations for identifying key events and understanding their role in the MOA

Here, we build on the work of Julien et al. (2009) and Andersen et al. (2014) to develop the Q-KEDRF. The following definitions are used in the Q-KEDRF:

Box 2. Overview of the Q-KEDRF toolbox.

Tabular Methods
- Application Scheme for IPCS Human Relevance Framework (Figure 1 in Andersen et al., 2014)
- Comparison of Proposed MOAs (Table 4 in Corton et al., (2014))
- Qualitative Species Concordance Table (Table 4 in Elcombe et al., (2014)
- Qualitative MOA Concordance across Chemicals (Table 5 in Corton et al. (2014)

Graphical Methods
- Flow chart of each proposed MOA (Figure 7; Figure 2 in Corton et al. 2014; Figure 2 in Budinsky et al., 2014)
- Dose-Response Arrays (Figure 8)
- Quantitative Species Concordance Table (Table 3; Table 5 in Budinsky et al., 2014)
- 3D Plotting for Visualizing KEs in Dose and Time (Figure 8 in Budinsky et al., 2014; Figure 6 in Corton et al., 2014)

Quantitative/Computational Methods
- Dose-Response Modeling (BMDS, Graphpad Prism, Other tools)
- Use of Dose Surrogates (AUC, Enzyme Induction levels, etc.)
- Dose-Response Slope Analysis (Tables 6 and 7 here)

- Key Event (KE): An empirically observable causal precursor step to the adverse outcome that is itself a necessary element of the MOA. KEs are necessary but usually not sufficient for the adverse outcome in the absence of other KEs.
- Associative Events (AEs): Biological processes that by themselves are not KEs in the hypothesized MOA but may serve as reliable indicators or biomarkers for KEs. AEs can be used as surrogates or biomarkers for a KE in a MOA evaluation; depending upon the nature of the biomarker, AEs may reflect exposure to a xenobiotic, the resulting effect, or both.
- Modulating Factors (ModFs): Biological and individual factors, including control mechanisms or host factors, that can modulate the dose–response relationship of one or more KEs, thus altering the probability or magnitude of the adverse outcome (Figure 2, Box B4).

AEs can easily be thought of as biomarkers. In this regard, their relationship to KEs may need to be explored, especially if the AE is needed to measure the KE (IOM 2010).

ModFs may alter the dose-response of the KE in a variety of ways. A selection (not inclusive) of ModFs in humans is provided in Table 1.

Both the KEDRF and Q-KEDRF represent an evolution of the MOA/HRF. Thus, both frameworks assume that sufficient evidence exists to posit the MOA under consideration and to identify hypothesized KEs based on this evidence (Boobis et al. 2006, 2008, 2009, Meek 2008, Meek et al. 2003, Seed et al. 2005, Sonich-Mullin et al. 2001).

If a putative MOA cannot be established, then the Q-KEDRF will not be applicable. Nonetheless, a risk assessment, albeit bearing greater uncertainty, can still be attempted using

other methods such as margin of exposure evaluation based on the most appropriate endpoint (Figures 1 and 2, Box B1).

A sequence of KEs represents a progression over both dose and time. Knowing the relationship between the various KEs in both dose and time along with an understanding of the underlying biology will contribute to the understanding of the role of particular KE within the MOA. Often, counterfactual information is not available. It may be very difficult to demonstrate the necessity of a particular proposed KE. Understanding the biology can help, but conclusive support of necessity will be a data gap.

Identifying a KE is based on the confidence one has that this event is necessary for the apical event/adverse outcome and is based on an overall WoE evaluation of qualitative and quantitative aspects of the MOA as well as whether the hypothesized roles of the KEs are consistent with the biological basis of the adverse outcome.

The Hill considerations have been adapted for use in understanding MOA. Hill (1965) termed these "viewpoints" or "features to consider" rather than true criteria. Hill's considerations are emphatically not a checklist and necessitate rigorous scientific thinking. They have been quite correctly called "guideposts on the road to common sense" (Phillips and Goodman 2006). Hence, the Key Event/Dose-Response Concordance analysis or Dose-time Concordance analysis requires a rigorous and reasoned WoE approach to reach an understanding of the overall MOA (Phillips and Goodman 2004). Very recently, newly evolved rank-ordered Bradford Hill considerations for application in a MOA analysis were developed (Meek et al. 2014a). In rank order, these include biological concordance, essentiality of key events, concordance of empirical observations, consistency and analogy.

For each proposed KE, if removal or blockade of its occurrence could be accomplished (*i.e.*, the counterfactual experiment), then its necessity (or lack thereof) and consequent identity as a KE could be supported. This is the consideration of essentiality. A cause–effect relationship between a chemical and an adverse effect can never be unequivocally proven because causality itself cannot be proven—only inferred with varying degrees of certainty (Adami et al. 2011). A proposed MOA represents a testable hypothesis (Popper 1959) and the KEs as aspects of that testable hypothesis can be examined in a weight of evidence framework to infer causality (Guzelian et al. 2005, Hill 1965, Phillips and Goodman 2004, 2006, Susser 1986).

Therefore, as indicated in earlier publications on MOA, an essential aspect of the process is identification and evaluation of attendant uncertainties. Each step in a MOA analysis should be accompanied by a list of critical and associated data gaps, with a clear indication of those, if filled, likely to have the most impact on the conclusions. The implications of the existing uncertainties should be explored during dose-response assessment.

Relationships between key events, AEs, and the adverse outcome

The development of a proposed or hypothesized MOA will necessitate identification of KEs and understanding of the dose-response and temporal relationships between the various KEs and the adverse outcome as well as between the KEs

Table 1. Modulating Factors (ModFs) potentially affecting KEs for dose-response in humans. ModFs fall into three general categories shown in the left column. The middle column shows subcategories and the right hand column shows some aspects to consider.

Category	Sub-category	Aspects
Host Factors	Genetic Variation	Polymorphisms
	Disease/Illness	Chronic
		Acute
	Defense mechanisms	Immune responsiveness
		DNA repair
		Cell proliferation
		Cell death
	Physiology	Sex
		Life stage
		ADME
		Hormonal status
Life Style	Diet	Calories
		Fat content
	Tobacco	Usage
	Alcohol	Usage
	Exercise	Frequency
		Intensity
	Pharmaceuticals	Usage
	Illegal drugs	Usage
	Dietary supplements	Vitamins
		Anti-oxidants
Environment	Co-Exposures	Duration
		Air
		Water
		Food
		Dust
		Occupational

Table 2. Dose-time concordance table for dimethylarsinic acid.

Table —Dose-Time Concordance					
Time	2 weeks	2–3 weeks	10 weeks	25 weeks	104 weeks
Dose (ppm in diet)	Increasing time ⟶				
2	Metabolism*	Metabolism*	Metabolism* Cytotoxicity	Metabolism* Cytotoxicity*	Metabolism* Cytotoxicity*
10	Metabolism*	Metabolism* Cytotoxicity	Metabolism* Cytotoxicity	Metabolism* Cytotoxicity*	Metabolism* Cytotoxicity*
40	Metabolism*	Metabolism* Cytotoxicity	Metabolism* Cytotoxicity Proliferation Hyperplasia	Metabolism* Cytotoxicity* Proliferation* Hyperplasia	Metabolism* Cytotoxicity* Proliferation* Hyperplasia Carcinomas
100	Metabolism*	Metabolism Cytotoxicity Proliferation Hyperplasia	Metabolism Cytotoxicity Proliferation Hyperplasia	Metabolism Cytotoxicity* Proliferation Hyperplasia	Metabolism* Cytotoxicity* Proliferation* Hyperplasia Carcinomas

The asterisk means that the key event has not been observed at the specific dose/time point but is presumed to have occurred. Although not used here, shading of the table may be helpful with a shading scheme based on the number of KEs. Figure 5 in Meek et al. (2014b) provides another organizational scheme for the dose-time concordance table (Please see Figure 3 for the MOA and text for details).

themselves. This is the purpose of the Dose-Time Concordance table (Table 2). Such a table also addresses the temporal aspects of Box B3 in Figure 2 (Meek et al. 2014b) .

In 2005, EPA's Office of Pesticide Programs proposed a MOA for the carcinogenesis of dimethylarsinic acid or DMA^V, also known as cacodylic acid (USEPA 2005c). DMA^V administered in the diet or drinking water produced bladder cancer in rats. There are four KEs in the MOA for bladder tumors in rats; these are: (1) generation of the reactive metabolite trivalent DMA (DMA^{III}) that is dependent on DMA^V and can be observed as the urinary excretion of trivalent DMA greater than 0.1 μM in urine; (2) cytotoxicity occurring within the superficial epithelial layer of the urinary bladder; (3) consequent regenerative proliferation; and, (4) hyperplasia of the urothelium (Cohen et al. 2006, USEPA 2005c). The qualitative relationships between these KEs in both dose and time is shown in Table 2, which is an example of the dose–time concordance table (Meek et al. 2014a, Meek et al. 2014b).

In two-year bioassays, dietary administration of 9.4 mg/kg/d DMA^V produced a statistically significant incidence of tumors; dietary administration of 4.0 mg/kg/d produced a statistically significant incidence of hyperplasia. There were no histopathological changes in the urothelium observable using light microscopy from dietary administration of 1 mg/kg/d or lower. In shorter term mechanistic studies using light and scanning electron microscopies to detect superficial cytotoxic changes, evidence of cytotoxicity was present at dietary doses of 1 mg/kg/d and higher. These same mechanistic studies used bromodeoxyuridine (BrdU) labeling index to assess cell proliferation and observed an increase in proliferation at a dietary dose of 1 mg/kg/d and above.

In rats administered DMA^V in drinking water, genomic microarray analysis revealed a change in the pattern of altered gene expression between 0.4 and 4.7 mg/kg/d, the same level at which an apparent threshold was observed using transmission electron microscopy (Sen et al. 2005). Critical cytotoxic urinary levels of the reactive metabolite DMA^{III} were present in rats

orally administered DMA^V at doses of 1 mg/kg/d and above, but absent at 0.2 mg/kg/d. The level of detection for DMA^{III} in urine was 0.01 μM (USEPA, 2005c).

Evidence strongly suggests that DMA^{III} is not DNA reactive, and likely is not genotoxic except at relatively high concentrations (Cohen et al. 2006). Table 2 summarizes the dose-response and temporal relationships for each of the KEs. For risk assessment purposes, it is reasonable to base the assessment on the most sensitive of the KE changes, that is, cytotoxicity. Based on such an analysis, the no-observed-adverse-effect level (NOAEL) is 0.2 mg/kg/d via diet. Similar findings have been identified in rats administered DMA^V in the drinking water (Cohen et al. 2006). Table 3 shows an example of the Dose-Response Species Concordance table that supports quantitative interspecies extrapolation of KEs.

Although the dose-response for humans in Table 3 is lacking, toxicokinetic interspecies extrapolation could be based on differences in the metabolism and kinetics of DMA^V in rats and humans. The evidence indicates that DMA^V is a poor substrate for the methylating enzyme for arsenicals in humans (As^{3+} methyltransferase, As3mt) whereas in rats, this enzyme can readily methylate DMA^V to trimethyl arsenic oxide (Thomas 2007). A physiologically based pharmacokinetic (PBPK) model for DMA^V could support further refinement of the risk assessment, but such a model was not fully developed in 2005 (Evans et al. 2008, USEPA, 2005c). In vitro cytotoxicity assays utilizing rat urothelial cells showed an effect at concentrations of approximately 0.2 μM or higher; in comparison, in vitro human urothelial cells showed less sensitivity, with cytotoxicity produced at concentrations of 0.5 μM and higher (Cohen et al. 2006). Hence, overall, humans would be less susceptible than rats based on both kinetics and dynamics. These quantitative differences could potentially be used to develop a data-derived species extrapolation factor or chemical-specific adjustment factor (USEPA 2011, WHO-IPCS 2005, Meek et al. 2014b).

Table 3. Dose-Response Species Concordance Table for Key Events (KEs) in the MOA of dimethylarsinic acid (DMA^V) (Adapted from USEPA; 2005c).

Event or factor	Qualitative concordance			Quantitative concordance and quantitative Dose-response		
	Animals	Humans	Concord-ance	Str.*	Animals	Humans
Key events						
Key Event #1 Metabolism to DMA^{III}	DMA^{III} detected in urine following 26 weeks treatment with 100 ppm DMA^V	Evidence following DMA^V exposure too limited to draw conclusions, but DMA^{III} shown to be present following human exposure to iAs	Plausible	+/−	Hill Model Fit K_d = 8.34, n = 1.07 (DMA^{III} in urine at 3 weeks (µM) vs Dose of DMA^V (mg/kg/d))	NA
Key Event #2 Urothelial Cytotoxicity	Urothelial toxicity observed in vivo in rats at 2 ppm but not enough for successive key events	Potential to occur in humans but unknown if sufficient DMA^{III} formed	Plausible	+/−	Frequency of Cytoxicity vs Dose of DMA^V (mg/kg/d); • 3 weeks, ▲ 10 weeks; BMD$_{10}$ at 3 weeks − 0.66, BMD$_{10}$ at 10 weeks = 0.02	NA
Key Event #3 Urothelial Proliferation	observed at 0.5 mg/kg/d DMA^V	Potential to occur in humans but unknown if sufficient DMA^{III} formed	Plausible	+/−	Proliferation at 10 weeks (BrdU Labelling Index) vs Dose of DMA^V (mg/kg/d); BMD$_{10}$ = 0.65	NA
Key Event #4 Hyperplasia	observed at 2 mg/kg/d or 0.3 to 2 µmol DMA^{III} in urine	Potential to occur in humans but unknown if sufficient DMA^{III} formed	Plausible	+/−	Frequency of Hyperplasia at 10 weeks vs Dose of DMA^V (mg/kg/d); BMD$_{10}$ = 1.36	NA
Apical Event Tumors	observed at 5 mg/kg/d DMA^V or 0.8 to 5.05 µmol DMA^{III} in urine	No data in humans	Concordance cannot be made because there is no human data	—	Frequency of Bladder Tumors (2 yr) vs Dose of DMA^V (mg/kg/d); BMD$_{10}$ = 1.91 (High Dose omitted from fit)	NA

*Str. = strength.

In such a case, this information could be added to the Dose-Response Species Concordance Table.

Low protein or vegetarian diets decrease the availability of S-adenosyl methionine (SAM), and arsenic methylation uses SAM as a methyl donor. Hence, diet may constitute a ModF to be considered (Gamble and Hall 2012).

The risk assessment conducted by EPA's Office of Pesticide Programs (OPP) used a benchmark dose lower confidence limit of 0.07 mg/kg/d DMA^V based on cell proliferation as the 1% point of departure (USEPA 2005c) and a nonlinear low-dose extrapolation to develop a reference dose protective of cancer based on this MOA. Here, this example serves to demonstrate the use of the Dose-Time Concordance Table (Table 2) and the Dose-Response Species Concordance Table (Table 3). The BMD information for KEs occurring at 10 weeks—cytotoxicity, proliferation, and hyperplasia—provided a way to order these KEs and supports their order in the dose–time concordance table (Table 2).

An example of how to use the RISK21 exposure-toxicity matrix is provided (Figure 3). The heavy dotted line on the matrix represents a hazard quotient (HQ) of one. The blue square represents the intersection of exposure and toxicity. If any part of this area extends above the line representing an HQ of one, then exposures may be of concern. In the case of cacodylic acid, all exposure levels within the range of chronic dietary exposures are less than the RfD (USEPA 2006). The exposure-toxicity matrix is flexible; in addition to the range shown here, probability distributions of exposure and/or toxicity can be shown as a means of visualizing probabilistic characterizations of exposure, toxicity, and risk.

Concordance of the MOA between humans and animals

The human relevance of a hypothesized MOA may depend on both qualitative and quantitative factors. As evident from the example with DMA^V above, EPA's Office of Pesticide Pro-

Figure 3. Use of MOA in the HESI RISK21 Matrix. Left: MOA for Tumor Induction by Dimethylarsinic Acid (DMA^V; Cacodylic Acid) that includes cytotoxicity, regenerative proliferation, and hyperplasia. This MOA is used to illustrate the dose-time concordance table and dose-response species concordance table (Tables 2 and 3). Right: Matrix showing the exposure estimates and toxicity range (BMDL$_{10}$ to RfD) for chronic dietary exposure, data from EPA, 2006.

grams clearly recognizes this fact and the need for assessing both qualitative and quantitative concordance of KEs between animals and humans (Dellarco and Fenner-Crisp 2012). For example, in the early 1990s, a technical panel from EPA concluded that male rat renal tubule tumors from chemicals that induced accumulation of α_{2u} globulin were likely not relevant to humans based on qualitative considerations (Rodgers and Baetcke 1993). Naphthalene produces respiratory tract tumors in rats, but the MOA for these tumors in rats is based on metabolic enzyme activity that is not present in humans (Piccirillo et al. 2012).

The Dose-Response Species Concordance Table (Table 3) is a means of illustrating the similarities and differences in a proposed MOA between humans and the test-species. Likely other information, narrative and/or additional tables, will be needed to provide all the information needed for species extrapolation.

Qualitative concordance of key events between humans and animals

Human relevance of the apical endpoint is best determined using a hypothetico-deductive WoE approach (Boobis et al. 2006, 2008, Meek et al. 2003, Rhomberg et al. 2010, Seed et al. 2005, Sonich-Mullin et al. 2001). To address human relevance of the MOA, qualitative concordance between humans and animals for each KE needs to be considered. In vitro data from human or animal cells or tissues and/or in silico data may also be available; these data play a useful role in the determination of concordance as well. Ideally, the data will be sufficient to determine which of the KEs is relevant to humans, and these data may thus be used to support statements about the relevance to humans of the hypothesized MOA in animals.

Quantitative concordance of the MOA between humans and animals

Quantitative examination of both the dose-response and timing of KEs is also necessary to determine human relevance. For example, a MOA may be operative in both animals and humans, but extremely unlikely in humans because of quantita-

tive toxicokinetic or toxicodynamic differences. If the KE has the potential to occur in humans, then this quantitative examination can be used to inform animal-to-human extrapolation. Hence, the quantitative concordance should provide information about the EC_{50} and/or point-of-departure values for as many KEs as possible in both humans and the animal test species. Including NOAELs or other measures of the no-effect level/threshold such as that defined using the EC_{05} baseline projection method of Silkworth et al. (2005) or the "hockeystick" fitting method of Lutz and Lutz (2009) may also be useful.

The role of quantitative dose-response information

For dose-response assessment, it can be extremely useful to examine quantitative dose-response information from as many relevant sources as possible (e.g., human, laboratory animal or in vitro data). These data will help inform the progression of events within the MOA. In vitro to in vivo extrapolation (IVIVE) may be necessary to express the dose-response for in vitro data on a similar dose scale as the in vivo data. Where possible, the actual dose-response plots should be shown. It is often helpful to show the dose-response of a KE and that of the apical event or adverse outcome on the same plot (e.g., Figure 2 in Simon, et al 2009). Once the MOA for rat liver tumor promotion by TCDD was considered, the task of arranging the dose-response plots in a figure that displayed the MOA in a meaningful way became easy. Rodent liver tumor promotion is one of the longest and most intensively investigated MOAs in toxicology (Budinsky et al. 2014). Developing similarly informative figures may not be as easy for less well-studied chemicals. Figure 8 is an attempt to create a similar figure for the uterotrophic response. For clarity, it is helpful to have the same dose range on the x-axis in all the plots. When not possible to provide plots of dose-response curves, sufficient narrative should be presented to explain animal/human similarities and differences. If sufficient data in both dose and time are available for a particular KE, a three-dimensional graph with an interpolated surface plot that shows the occurrence of the KE along both dose- and time-axes may be very informative (Box 2; Budinsky et al. 2014).

Use of dose-time and dose-response concordance information in understanding the MOA

In general, events that occur at low doses and/or at early stages in the progression toward the apical event may represent:

- the start of a temporal progression;
- the initial stages of a developing change; or,
- a factor that potentially causes other KEs that occur at higher doses or at a later time in the progression.

Generally, demonstrating that a particular event is necessary is experimentally difficult; yet, it may be possible in some cases (*e.g.*, with transgenic or knockout animals), thus providing a powerful counterfactual demonstration supporting the identification of the event as a KE (Phillips and Goodman 2006). In the example used in Table 2 and Figure 3, let us assume that blocking metabolism of DMA^V or cacodylic acid to dimethyl arsinous acid (DMA^{III}) could reduce or alleviate the KE of urothelial cytotoxicity. The enzyme arsenic methyltranferase (As3mt) catalyzes all steps in the metabolic pathway from arsenite to mono, di, and trimethylated arsenic compounds (USEPA 2005c). If cytotoxicity and tumors did not occur when As3mt was inactivated, this would confirm the role of metabolism and resulting cytotoxicity as necessary and thus as KEs; conversely, if cytotoxicity and tumors occurred even when As3mt was inactivated, one could no longer support the identification of metabolism and cytotoxicity as KEs. Once the DMA^{III} is formed, it readily reacts with free sulfhydryl groups. Co-administration with high doses of a sulfhydryl-containing chemical, such as dimercaptopropanesulfonic acid (DMPS) can act as a trap for the DMA^{III}, reduce or prevent its reaction with proteins, and thus reduce or prevent its biological effects. Co-administration of DMA^V with DMPS inhibits the induction of cytotoxicity and regenerative proliferation of the urinary bladder, providing evidence for DMA ^{III} as the reactive intermediate and AE/KE in the DMA^V-induced bladder cancer in rats (Cohen et al. 2006).

The exact nature of a KE cannot be necessarily understood from either its dose-response or its timing of occurrence. For example, some early KEs may need to be sustained in order for later KEs or the apical event/adverse outcome to occur (*e.g.*, Budinsky et al. 2014).

Toxicokinetics may affect this timing. For example, lipid soluble chemicals may be stored in adipose tissue for months or years and produce effects on an ongoing basis; for similar reasons, the dose of a bioaccumulative chemical may be measured as body burden or tissue concentration. In such a case, the area under the curve (AUC) in units of concentration × time would likely represent the ongoing accumulation in both dose and time better than body burden or tissue concentration at a single time point. Sequestration of a chemical by protein binding may also be represented best by the AUC. A monotonic dose–response relationship between the AUC and a biomarker for a putative KE such as enzyme induction indicates that exploring the quantitative relationship between this biomarker and the apical event/adverse outcome may likely help elucidate details of the MOA.

In other cases, the occurrence of some early KEs may trigger a cascade of other events. These early KEs either resolve themselves or are no longer empirically observable. However, the cascade of triggered events continues and leads ultimately to the adverse outcome/apical event. An example of such an effect is illustrated by the difference between long-acting and short-acting estrogens; short-acting estrogens produce early but not late events in the uterotrophic response whereas long-acting estrogens produce both. Estradiol, a long-acting estrogen, can stimulate uterine growth for up to 72 hours whereas the effects of estriol, a short-acting estrogen, last only 24 hours. In fact, estriol and other short-acting estrogens may display partial antagonism when continuously administered in longer-term assays (Clark and Markaverich 1984). Again, these various estrogenic compounds show differences in their dose-response over time.

The Q-KEDRF toolbox

Quantitative methods are often a good way to understand modulating factors. When a sufficient number of experiments determine the procession/cascade of KEs on both dose- and time-scales, quantitative methods are less necessary to obtain an understanding of the MOA. In such cases, the Dose-Time Concordance Table will suffice, and such was the case for DMA.

The relationship of KEs to the critical effect/apical or adverse outcome can be understood by expressing the tumor BMD as a multiple of the BMD values of various KEs (*e.g.*, Simon et al. 2009). BMD_{10} values are shown on the figures in Table 3. Values for the BMD multiple for the three KEs, cytotoxicity, proliferation and hyperplasia, can be determined as:

$$\text{BMD Multiple} = \frac{\text{BMD}_{ApicalEvent}}{\text{BMD}_{KeyEvent}} \qquad (1)$$

Using Eq. (1), one can determine that the tumor POD is almost 100 fold greater than the BMD_{10} for cytotoxicity at 10 weeks, about 3 fold greater than the BMD_{10} for proliferation at 10 weeks, and about 1.5 fold greater than the BMD_{10} for hyperplasia at 10 weeks. These values provide a means of judging the relative position of the various KEs along the dose continuum.

Quantitative dose-response methods also may prove very useful for understanding and refining proposed MOAs. For example, Simon et al. (2009) used both potency and steepness to determine the dose progression of likely KEs in the MOA for rodent liver tumorigenesis by dioxin. This approach was used again to examine nuclear receptor activation leading to tumor promotion (Budinsky et al. 2014, Corton et al. 2014).

While no single method is appropriate for all situations, the methods described in this section are all part of the Q-KEDRF toolbox. Contrast tests and regression analysis using well-established statistical methods may prove useful for ordering events within a hypothesized MOA (Bretz et al. 2005, Sawilowsky 2002, Tukey et al. 1985). Lutz and Lutz (2009) provide full details of their "hockey stick" model and an R script for ease of use. For developing dose levels corresponding to specified response levels (i.e., benchmark doses), Murrell et al. (1998) suggest the use of the calculated slope of the dose-response and baseline projection. Silkworth et al. (2005) implemented a form of this method but did not describe details of their calculation. The method was fully developed, including

calculation of confidence intervals in Budinsky et al. (2010). Sand et al. (2006) used the second and third derivatives of the dose-response function to obtain a "transition dose range." Further, they identified a response level of 21% as the transition point for the Hill model.

Naïve practitioners may be tempted to use of the numerical results of a single method as a quantitative threshold. In this regard, any quantitative estimate of a threshold needs to be considered in the light of biological significance, and quantitative estimates of thresholds and transitional dose values (TDVs; see Section 4 below) from a variety of methods should be developed (Budinsky et al. 2010). The discussion of thresholds in Slob and Setzer (2014) is particularly enlighting. Notable is their argument that dose is better represented on a logarithmic scale than on a linear one. The use of logarithms with dose is consistent with thermodynamic principles (Waddell 2005, Waddell 2008). This caveat notwithstanding, the ability to obtain quantitative dose values within the low-dose region can greatly help determine the order in dose and time of events within a hypothesized MOA (See Supplementary Content for an example).

Modulating factors—accounting for variation within the human population

The application of the MOA/HRF and the QKEDRF can provide informative and quantitative descriptions of the MOA and dose-response for adverse outcomes (cancer and non-cancer) including those at low, environmentally relevant exposure levels. Such an approach is essentially designed to describe the form of the dose-response curve for a generalized population. What is also needed is an approach that allows for incorporation of the influence of ModFs on the dose-response of KEs that will ultimately enable the quantitative population-level assessment of risk at low exposure levels. ModFs should be understood in terms of their effects on biological processes and KEs within an MOA. The effect of a low protein vegetarian diet on the availability of S-adenosyl methionine as a possible ModF for the toxicity of DMA^V has already been discussed.

One universal ModF is likely to be individual variation in reserve capacities, for example, differing amounts of reduced glutathione that affect the occurrence of particular KEs between individuals and over time within a single individual. Other examples would be the expression of the p53 gene product or the occurrence of oxidative DNA damage.

Variations in the intracellular level of a large number of transcription factors and cofactors can alter both the efficacy and potency for both steroid and glucocorticoid hormones (Blackford et al. 2012, Simons 2010, Sun et al. 2008, Zhang et al. 2012). In fact, limitations in the amount of coregulatory proteins available within the transcription complex may lead to non-monotonic dose response curves such as squelching (Charlier 2009, Kraus et al. 1995, Zhang and Teng 2001). Graphical analysis of these changes yields valuable mechanistic information when the production of the apical response follows a first-order Hill dose-response curve (Dougherty et al. 2012, Ong et al. 2010, Simons and Chow 2012). However, regardless of the order of the dose-response curve of the adverse outcome/apical response, the magnitude and/or position of the dose-response curve will likely be similarly

modified by any chemical that binds to nuclear receptors and/or other transcription cofactors.

There may exist many potential ModFs for any particular exposure scenario (*e.g.*, specific chemical, type of exposed individual or group). Therefore, organizing these factors based on common biological mechanisms would be helpful. By doing so, the likelihood of a ModF affecting a particular MOA could be determined. One approach described here is to identify a list of general ModFs that can be broadly separated as Host, Life Style and Environment (Table 1). Other classification schemes for ModFs, perhaps based on MOA, will likely emerge as risk assessment practitioners gain experience with the Q-KEDRF. The OECD is currently developing a program on AOPs, and the International QSAR foundation is developing an "Effectopedia" to provide information about AOPs/MOAs as part of a global scientific collaboration; the Q-KEDRF will likely interface quite well with these efforts (Ankley et al. 2010, Patlewicz et al. 2013). The use of the term "Initial Molecular Event" (IME) to refer to the first step Event, as suggested by Patlewicz et al. (2013), is appropriate and conveys an accurate message—that the initial event may not obligatorily lead to the adverse outcome.

Examples of modulating factors

Two examples are presented below with the goal of improving the understanding of how ModFs can affect KEs and potentially impact the dose-response for the adverse outcome. These examples illustrate different aspects of KEs within biological pathways: xenobiotic processing (metabolism) and endocrine stimulation.

Example 1: Genetic variation in PON1 potentially modulates chlorpyrifos metabolism and toxicity

The MOA for OPs is well known—inhibition of cholinesterases with toxicity manifested as central and peripheral cholinergic effects (Figure 4) (Mileson et al. 1998). Cholinesterase inhibitors include carbamate insecticides, physostigmine used to treat glaucoma, and Δ^9-tetrahydrocannabinol, the active moiety in marijuana. Paraoxonase 1 (PON1) is an arylesterase that metabolizes organophosphate compounds (OPs). Thionophosphorus OPs such as chlorpyrifos (CPF) are metabolized to the oxygen analog or oxon by CYP450 mixed function oxidases. These oxons are potent inhibitors of acetyl cholinesterase (AChE). CPF oxon is inactivated by PON1 in the liver and other tissues (Smith et al. 2011, Timchalk et al. 2002a; 2002b).

Host factors—genetic variability and lifestyle factors. In humans, PON1 activity is age-specific, increasing about 3.5 fold between birth and 7 years of age, remaining constant thereafter (Figure 5) (Smith et al. 2011). Genetic polymorphisms exist in the coding regions of PON1 gene with consequent variation in catalytic activity. For example, PON1 polymorphism at amino acid 192 [glycine (Gln; Q allele) to arginine (Arg; R allele) substitution] changes PON1-mediated esterase activity depending on the substrate present (Adkins et al. 1993). PON1 (R192) hydrolyzes CPF oxon more efficiently than PON1 (Q192) (Richter et al. 2009). Along with the general increase in activity with age, differing phenotypes mature at different rates (Huen et al. 2010). Polymorphisms exist in the
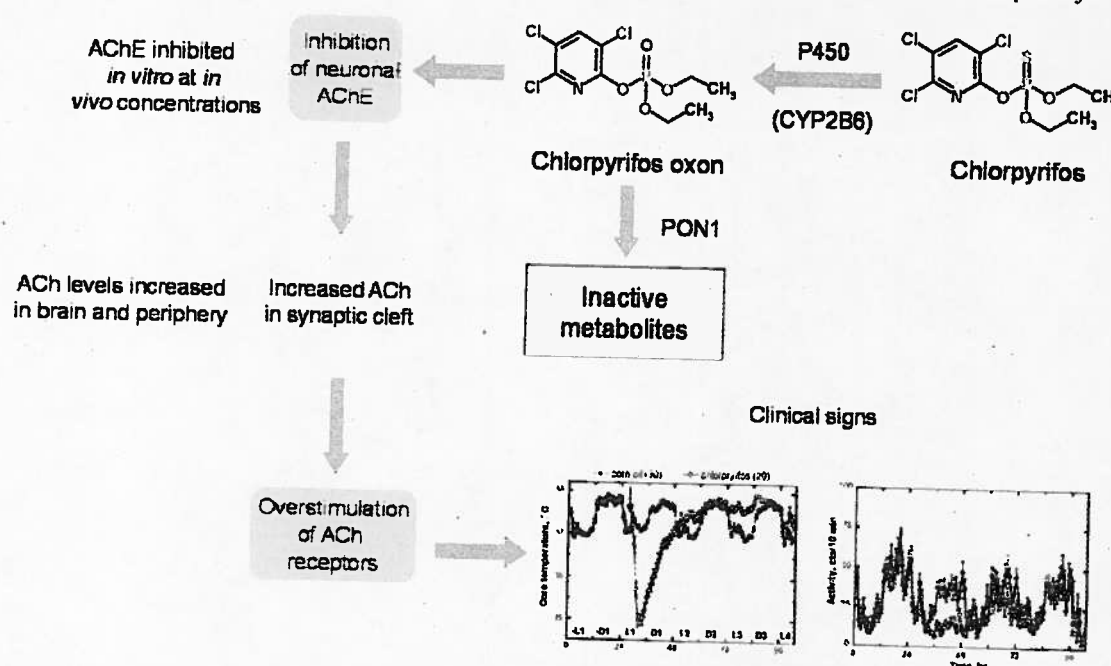
Figure 4. Mode of Action of Chlorpyrifos showing metabolic activation to CPF-oxon and inhibition of acetylcholinesterase as the critical effect. (Figure courtesy of Dr. Alan Boobis).

promoter region of PON1 and may affect expression level and tissue activity. A single nucleotide polymorphism located 108 bases before the transcription start site (PON1$_{-108}$) accounts for 22.4% in the variability in arylesterase activity (Brophy et al. 2001, Deakin and James 2004). Overall, an individual's

Figure 5. PON1-mediated Vmax values vs. age (upper plot). PON1 functional phenotypes are represented by open circles, open triangles, and open squares for QQ, QR, and RR, respectively (see text for definitions). CPF- oxon hydrolysis V_{max} values in plasma over paraoxon hydrolysis activity (lower plot) resolves QQ and QR, but not QR and RR. (From Smith et al. 2011; permission to reproduce figures granted by Dr. Jordan Smith, 22 March 2013.).

PON1 activity is dependent on variations in the coding region as well as the promoter region. Both the polymorphisms and the age-dependent increase in activity would be categorized as host factors. The age-dependent increase in V_{max} in plasma PON1 activity on a plasma volume basis for individuals of all three genotypes (QQ, QR and RR) is shown in Figure 5.

In addition to these host factors, a number of lifestyle factors affect PON1 activity. Statins are cholesterol-lowering substances that occur naturally in red rice yeast and are also prescribed as drugs. In some human studies, very modest increases in serum PON1 have been observed in those taking statins. However, in other studies, no effect is seen (Costa et al. 2011). Moderate alcohol consumption appears to increase serum PON1 (Sierksma et al. 2002). Pomegranate juice contains several polyphenols and its consumption increases plasma PON1 activity in normal humans and in diabetic patients (Aviram et al. 2000, Rock et al. 2008). The lifestyle factors increase PON1 activity and would tend to desensitize individuals to the effects of thionophosphorus OPs.

Consideration of modulating factors in a chlorpyrifos risk assessment. For risk assessment purposes, the question that must be asked is whether changes in PON1 actually translate into changes in sensitivity, and, if so, whether these host and/or lifestyle factors produce sufficient variation in PON1 activity such that individuals with a sensitive phenotype such as QQ or the very young might constitute an at-risk subpopulation.

When workers exposed to CPF during manufacture were compared to a referent group of chemical workers, no effect of PON1 phenotype was observed (Albers et al. 2010, Garabrant et al. 2009). Urinary 3,5,6-trichloro-2-pyridinol (TCPy) is a metabolite of CPF and a specific biomarker of exposure (Alexander et al. 2006); TCPy levels in all exposed workers were less than those paralleling previously determined no-observed-effect levels for red blood cell (RBC) AChE inhibi-

Figure 6. Modeled chlorpyrifos pharmacokinetics in adults and children and resulting AChE inhibition in erythrocytes. A. RBC AChE inhibition from the AUC (left) and maximum CPF concentrations (right) in blood (from Hinderliter et al. 2011). B. Modeled time courses of CPF and CPF oxon in blood from dietary exposures (upper panel) and corresponding RBC AChE inhibition (lower panel). (Reprinted from *Regulatory Toxicology and Pharmacology* (Hinderliter, P.M., Price P.S., Bartels M.J., Timchalk C., Poet T.S. 2011. Development of a source-to-outcome model for dietary exposures to insecticide residues: An example using chlorpyrifos, Regul. Toxicol. Pharmacol. 61, 82–92) with permission from Elsevier.).

tion and changes in neurological function (Albers et al. 2004a; 2004b; 2004c; 2007, 2010, van Gemert et al. 2001).

Enzyme kinetics of PON1 were analyzed in liver microsomes and plasma in both children and adults to measure quantitative age-dependent differences (Smith et al. 2011). These data were incorporated into a probabilistic physiologically based pharmacokinetic and pharmacodynamic (PBPK/PD) model for CPF (Price et al. 2011, Timchalk et al. 2002a, Timchalk et al. 2002b). With this model, the relationship between urinary TCPy and either plasma butyrylcholinesterase (BuChE) or RBC AChE was determined and related to the exposure to CPF. Model results are shown in Figure 6. In three-year-old children, the greatest percent reduction in ChE levels for typical dietary intake was 0.001%. In addition, a sensitivity analysis of the PON1 parameter in blood and liver revealed

only a modest influence of this factor. The presence of other detoxification enzymes established a lower limit for the effect of PON1 variation (Hinderliter et al. 2011, Price et al. 2011).

In contrast, at a dose of 300,000 ng/kg/d of CPF, typical of a high-dose animal study, the model indicated that both the age-dependence and the polymorphisms in the activity of hepatic PON1 would be reflected by substantial differences in RBC AChE levels; however, neither these age-dependent differences nor PON1 enzyme polymorphisms are likely to affect RBC AChE levels at real-world human exposure levels (Garabrant et al. 2009, Hinderliter et al. 2011, Smith et al. 2011, Timchalk et al. 2002a; 2002b).

To incorporate ModFs into risk assessment, the effect of these factors needs to be considered at the point of departure or at current exposure levels and not in a purely

abstract way. An effect of human variation in PON1 on RBC AChE inhibition was observed in the model output at a dose of 300,000 ng/kg/d of CPF but not at current dietary exposures of children and adults for which the respective doses are estimated to be less than 11 ng/kg/d and 3.4 ng/kg/d. Increased sensitivity was not observed at dietary exposures because the exposures were too low to produce a biologically meaningful change in the activity of various cholinesterases, even in sensitive individuals. In addition, individuals of the RR phenotypes appear to have higher activity of PON1 in plasma, thus providing similar capacity for clearance (Figure 5; Smith et al. 2011). Therefore, while the presence of polymorphisms and the age-dependence of PON1 provide illustrations of potential ModFs, the actual effects of these factors must be considered in the context of the entire dose-response curve and relevant exposure levels.

This examination of the MOA for CPF-inhibition of AChE includes tiers 1 through 4 of toxicity resources in the RISK21 roadmap (Figure 1). *In vitro* and *in vivo* data from humans were included; a PBPK/PD model was used for IVIVE and the Q-KEDRF was used to evaluate the ModFs of age and genetic polymorphisms. This probabilistic model is an excellent example of the use of quantitative MOA information in a risk assessment.

Table 4 provides an example of the Species Concordance table for ModFs and presents some of the information discussed above. The table format is sufficiently flexible to accommodate both qualitative and quantitative information. Although the information for CPF was obtained from humans, the columns for animals represent placeholders for those situations in which species extrapolation of the effect of ModFs needs consideration.

Example 2: Factors that can modulate the uterotrophic response

Estrogens induce uterotrophy through activation of the estrogen receptor alpha (ERα), a ligand-activated nuclear receptor and transcription factor. Cellular and physiological factors can modulate the estrogen dose-response for ERα activation, subsequent KEs, and uterine weight gain, the latter considered to be the critical effect in this example. A positive uterotrophic response for a chemical indicates a potential for endocrine disruption (OECD 2003).

Progesterone opposes estrogenic effects and reverses estrogen-induced uterotrophy. Progesterone stops cell growth and prevents the uterine lining from shedding. Like estrogen, progesterone is a ligand that activates a transcription factor. All transcription factors require cofactors for transcription to occur. One function of these cofactors is to increase the activity of RNA polymerase II, sometimes by facilitating chromatin remodeling and RNA polymerase II access to transcriptional start sites. For constitutively expressed genes, chromatin remodeling plays a smaller role than other gene regulatory factors (John et al. 2011). In contrast, RNA polymerase II is already bound at the transcription start site of a large number of other genes and the binding of a transcription factor is the signal for the polymerase to "start" (Levine 2011). Cofactors that interact with both the estrogen and progesterone receptors include steroid receptor coactivator-1 (SRC-1), receptor interacting protein 140 (RIP140), and the histone acetyl transferase chromatin-binding protein/p300 (Kobayashi et al. 2010, Simons 2008, Simons 2010).

Among the mechanisms by which progesterone is proposed to antagonize estrogen actions is by binding to progesterone receptors (PRs) to form complexes that compete with ERαs for cofactors that help mediate and thus increase ERα-mediated gene transactivation (Giangrande et al. 2000, Kraus et al. 1995, Parisi et al. 2009, Wen et al. 1994). In general, the effects of progesterone oppose the effects of estrogen. Thus, the dose-response curve shifts to the right and the system or individual becomes less sensitive to the effects of estrogens. Given that estrogens induce synthesis of PRs, these combined effects may serve as a means of feedback inhibition of estrogen-activated responses.

Uterotrophy as a model system for understanding MOA. Estrogen-induced uterotrophy in rats is an extensively studied response that has been documented to proceed through estrogen binding to the intracellular ERα as the MIE and is a KE in the MOA for the uterotrophic response. The induction of several genes (i.e., ornithine decarboxylase, glucose-6-phosphate dehydrogenase, lactoferrin, c-fos, and uterine peroxidase) occurs in response to estrogen, and these gene expression changes have been proposed as KEs in the MOA of estrogen-induced uterine growth (Figure 7; OECD 2003). Microarray assays have identified various other genes that may also be part of the overall MOA (Heneweer et al. 2007, Naciff et al. 2003).

The effects produced by ModFs shown in Table 5 can modify gene function not only through direct effects on DNA and chromatin but also by altering the strength of the various

Table 4. Dose-response concordance table for Modulating Factors (MFs) in the MOA of chlorpyrifos.

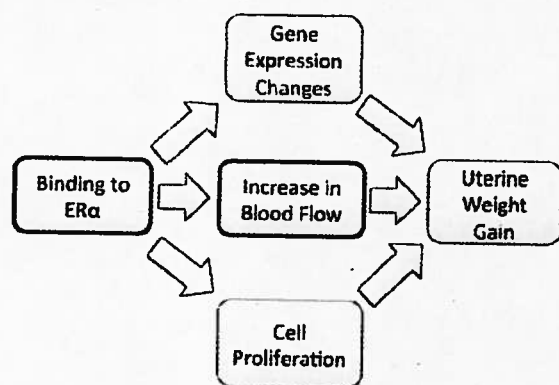| Event or factor	Qualitative concordance			Quantitative concordance and quantitative Dose-response		
	Animals	Humans	Concordance	Strength	Animals	Humans
Modulating Factors						
MF and affected KE	Animals	Humans	Concordance	Strength	Effects in Animals	Effects in Humans
MF #1 Genetic Polymorphism	NA	R vs. Q allele	NA			QQ genotype more sensitive, but at current exposure levels this difference is not a factor
MF #2 Use of Statin drugs	NA	Statins increase PON1 activity	NA			Statins modestly increase PON1 activity, but the effect is not consistently observed
MF #3 Alcohol Use	NA	Alcohol use increases PON1 activity	NA			This effect is likely not a factor at current exposure levels

Figure 7. Putative MOA for the uterotrophic response.

binding reactions occurring during gene transcription, including interactions between DNA and protein, between RNA and protein, between DNA and RNA, and between various proteins. The effect of these associations on dose-response is not clear at this time. However, the Q-KEDRF approach allows one to test the prediction that chemicals and factors with similar molecular targets will evoke comparable changes in the adverse outcome/apical event.

The rat uterotrophic response to estrogens was selected for a case study of the utility of using a MOA approach. The first step, of course, was to identify KEs or AEs that could serve as biomarkers for these KEs. Given the abundance of experimental data over the years for rat uterotrophy, this task was expected to be a relatively straightforward application of the new framework (Figure 2). OECD (2003) identifies binding to ERα as the MIE and provides a list of early and late events associated with uterotrophy. Unfortunately, dose-response and timing of these early and late events have not been obtained from the same species or preparation and thus, it is difficult to array these in a meaningful Dose-Time Concordance table. However, guidance from OECD as well as the scientific literature was used as the basis of a putative MOA and a set of proposed KEs for uterotrophy (Figure 7). Given the extent of investment in testing for endocrine effects and the relative maturity of the uterotrophic assay, the lack of information from the same or at least comparable studies seems surprising. This situation emphasizes the need to design studies that address the particular question at hand as it relates to elucidation of the MOA, and illustrates how effective the MOA framework can be in rapidly and effectively identifying critical data gaps. Consideration of MOA as early as possible in the risk assessment process would foster the collection of appropriate data to

Table 5. Cellular effects of modulating factors.

Effect	Sub-effect
Gene Structure	Mutations
	Deletions
	Duplications
Gene Expression	Transcription factors
	Co-activators/accelerators
	Co-repressors/decelerators
	Co-modulators
Post-translational modifications	Acetylation
	Methylation
	Phosphorylation
	Others

inform the MOA based on the expected value of the information (Meek et al. 2014a, Meek et al. 2014b). Such an approach would be entirely consistent with the method of problem formulation described in NRC (2009).

Following absorption of estrogen or an estrogenic chemical, binding to ERα would be the MIE. This binding has been measured in a number of species in vivo and in cell-free preparations (Levin et al. 1993, Notides et al. 1981). Following receptor binding, early events would include (1) altered expression of estrogen sensitive genes; (2) an increase in uterine blood flow; and (3) an increase in cell proliferation. Respectively, these events can be measured by: (1) microarrays or qRT-PCR; (2) flow transduction or weight gain; and (3) mitotic index or BrdU labeling. Because of the lack of sufficient data from a single high-quality study, as already stated, it is difficult to determine the exact role of these putative KEs in the MOA, but assessing the whole body of evidence using a WoE analysis, KEs can be substantiated. The apical event is, of course, uterine weight gain. At the present time, the order and timing of the changes shown in the third and second columns of Figures 7 and 8, respectively, are not known (Ashby et al. 1999, Gorski et al. 1977, Heneweer et al. 2007, Kaye et al. 1971, Naciff et al. 2003, OECD 2003).

At this point, conclusive identification of putative KEs becomes difficult due to: (1) variations in experimental systems; (2) the absence of data representing multiple KEs from the same study or same laboratory; and (3) and insufficient data points to make quantitative conclusions about dose-response.

Identification of key events for uterotrophy using WoE. Absorption is considered part of absorption, distribution, metabolism, and excretion, and is thus not identified as a KE, although it is the initial event in the process. For some chemicals, metabolic transformation that occurs close in time to absorption may either bioactivate these chemicals to toxic/active metabolites (e.g., polycyclic aromatic hydrocarbons/tamoxifen and cortisone, respectively) or detoxify/inactivate them (e.g., CPF oxon/cortisol) (Chapman et al. 2013, Furr and Jordan 1984). Estrogenic compounds contain one or more phenol groups and, following oral exposure, may be inactivated before reaching the systemic circulation by first-pass phase II metabolism in enterocytes or the liver (e.g., Hengstler et al. 2011). Hence, for estrogenic compounds and uterotrophy, metabolic transformation would not be a KE; however, metabolism may be a KE for other substances that are transformed to toxic metabolites (e.g., dimethylarsinic acid).

For uterotrophy, the MIE of binding to ERα will be a KE if it is empirically observable, and it is very probable that cell proliferation is also a KE. Two KEs can actually be conclusively identified on the basis of counterfactual reasoning and are shown with a thicker outline of the event boxes in Figure 7. The basis for identifying binding to ERα as a KE is the fact that estrogen-receptor knockout mice do not show evidence of cell proliferation, that is, DNA synthesis, in response to estrogen (Curtis et al. 1996, Klotz et al. 2002). However, other responses associated with estrogen-induced uterotrophy such as water imbibition and lactoferrin induction are maintained in the absence of ERα (Das et al. 1997, Winuthayanon et al. 2010). The basis for identifying the increase in blood flow as a KE is the disruption
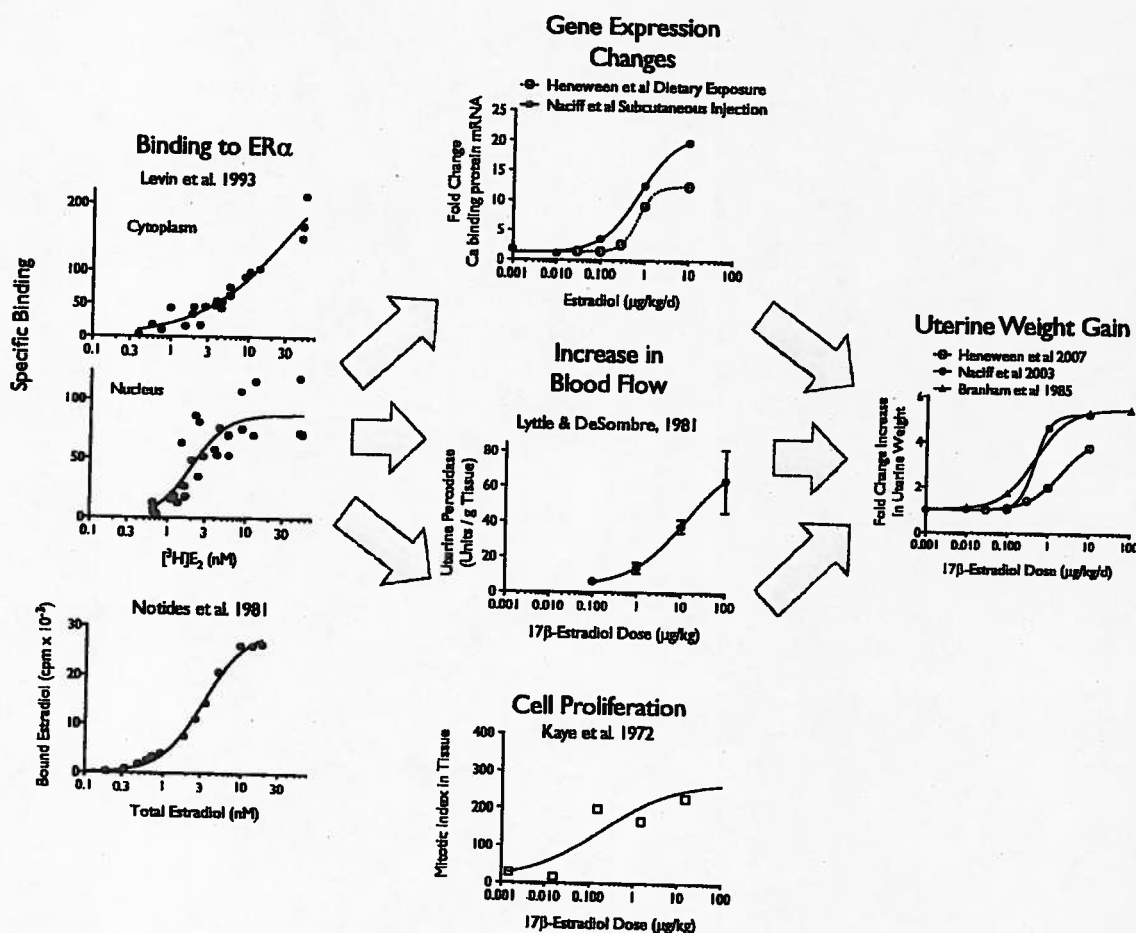
Figure 8. Dose-response plots for putative key events in the MOA for the uterotrophic response.

of the uterotrophic response by L-NG-nitroarginine methyl ester (LNAME) that blocks nitric oxide synthase (Rao et al. 1995, Rosenfeld et al. 1996). Alternatively, the production of catechol estrogens due to an estrogen-mediated increase in peroxidase may also contribute to alpha-adrenergic activation, vasodilation of the uterine arteries, and a consequent increase in blood flow (Lyttle and DeSombre 1977, Farley et al. 1992, Stice et al. 1987a; 1987b). In this example, the increase in uterine peroxidase is being identified as an AE to represent the increase in blood flow (Figure 8).

Dose-response modeling can elucidate the MOA for uterotrophy. Table 6 shows values for Hill model fits for the various responses of KEs and putative KEs. When data are available from a single study, both the EC_{50} and the slope of the dose-response curve are important in understanding the MOA and the relationship to the apical response (e.g., Simon et al. 2009).

EPA's Cancer Guidelines (USEPA 2005a) suggest the possibility of using an earlier KE as a precursor to the apical event and developing a toxicity criterion using the dose-response of this KE. Caution is warranted when using a KE as the basis for development of a toxicity criterion when the dose-response of the KE has a higher value of the Hill coefficient than the apical response; steeper dose-response curves (higher Hill coefficients) will have greater nonlinearity than a first-order Hill response and thus, the rising phase of the dose-response may commence at a higher dose value. Therefore, using the dose-response of the KE as the basis of a toxicity factor may not be a health-protective choice in the case of an apical event or critical effect known to follow a first-order Hill function, as is the case for uterotrophy (OECD 2003). By the same reasoning, the use of an early KE as the basis of a toxicity factor may be inappropriately over-conservative when the KE exhibits a shallower dose-response curve (lower Hill coefficient) than does the critical effect/adverse outcome.

The variation in the Hill coefficients observed in Table 6 is likely a reflection of the fact that these data were obtained from disparate sources. The plots of estrogen binding in the left column of Figure 8 were obtained *in vitro* and thus, IVIVE would be needed to set these on a similar dose scale as whole animal effects.

At this time, most available dose-response curves for estrogen-induced genes and other responses associated with uterotrophy have so few data points that the determination of quantitative aspects of dose-response becomes problematic. Even after all the years of studying uterotrophy, the shape of the curve for the critical effect of uterine weight gain has not been firmly established (Note the variation between the three curves in the rightmost plot of Figure 8).

For these reasons, even the relatively superficial MOA for uterotrophy cannot yet be constructed without new, more detailed data. First, high-quality dose-response curves with more data points for intermediate responses are critical so that an accurate determination of the position (*i.e.*, EC_{50}) and shape

Table 6. Quantitative aspects of the dose-response of key events in the uterotrophic response.

Key event	Study	Hill model parameters	Transition dose values	
			Starting points and slope-based TDVs (Murrell et al., 1998)	BMD_{21} as a transitional dose (Sand et al., 2006)
Binding to ERa	Levin et al. (1993) (cytosol) (fractional binding response)	$K_d = 31.2$ nM Log $K_d = 1.49$ $n = 0.76$	(13.8, 102.8) (5.8, 62.4) **1.26 nM**	**5.41 nM**
	Levin et al. (1993) (nucleus) (fractional binding response	$K_d = 2.04$ nM Log $K_d = 0.310$ $n = 1.94$	(1.49, 27.3) (2.85, 51.8) **1.05 nM**	**1.03 nM**
	Notides et al. (1981)[a] (fractional binding response)	$K_d = 3.25$ nM Log $K_d = 0.512$ $n = 1.61$	(1.853, 7.48) (4.99, 20.7) **1.11 nM**	**1.43 nM**
Gene expression changes in relation to uterine weight gain (Naciff et al., 2003)	Naciff et al. (2003) (fold increase in Ca binding protein)	Bmax = 22.82 fold $K_d = 0.807$ µg/kg/d Log $K_d = -0.0930$ $n = 0.755$	(0.1, 3.5) (1.0, 12.5) **0.082 µg/kg/d**	**0.140 µg/kg/d**
	Naciff et al. (2003) (fold increase in uterine weight)	Bmax = 5.48 fold $K_d = 1.26$ µg/kg/d Log $K_d = 0.010$ $n = 0.914$	(0.1, 1.12) (1.0, 4.63) **0.171 µg/kg/d**	**0.240 µg/kg/d**
Gene expression changes in relation to uterine weight gain (Heneweer et al., 2007)	Heneweer et al. (2007) (fold increase in Ca binding protein)	Bmax = 12.66 fold $K_d = 5.35$ µg/kg/d Log $K_d = 0.728$ $n = 1.54$	(0.3, 2.62) (1.0, 9.1) **0.290 µg/kg/d**	**2.26 µg/kg/d**
	Heneweer et al. (2007) (fold increase)	Bmax = 3.8 fold $K_d = 15.65$ µg/kg/d Log $K_d = 1.195$ $n = 1.191$	(1.0, 2.02) (10.0, 3.79) **0.220 µg/kg/d**	**5.15 µg/kg/d**
Cell proliferation	Kaye et al. (1971) (increase in mitotic index	Bmax = 276 figures $K_d = 0.809$ µg/kg Log $K_d = -0.092$ $n = 2.21$	(1.5, 166) (15, 227) **0.0073 µg/animal/d**	**0.444 µg/animal/d**
Increase in blood flow measure by uterine peroxidase	Lyttle and DeSombre (1977)	Bmax = 69 units/g $K_d = 17.3$ µg/animal Log $K_d = 1.24$ $N = 0.561$	(1.0, 13.8) (10.0, 37.6) **0.053 µg/animal/d**	**1.64 µg/animal/d**
Uterine weight gain	Branham et al. (1985) (mg wet weight)	Bmax = 5.4 fold $K_d = 1.85$ µg/animal/d Log $K_d = 0.268$ $n = 0.271$	(0.1, 1.73) (10.0, 5.31) **0.078 µg/animal/d**	**0.014 µg/animal/d**

Here, the results of two methods for determining transitional dose values (TDVs) are shown. Details of the calculation methods are provided in the text. The form of the Hill model used here is shown in Table 7.

[a]Notides et al. (1981) observe the Hill coefficient for E2 binding to cell-free preparations ERα varies with the concentration of the receptor (from $n = 1.1$ at 0.3 nM ERα to 1.6 at 3.0 and 4.8 nM ERα, indicating that the Hill coefficient increases with increasing concentrations of ERα.

(*e.g.*, first- or second-order Hill plot) of the curve is possible. This level of information is needed for all events being considered as KEs. These data would be invaluable in eliminating proposed KEs for which the parameters of the dose-response curve are not compatible with those of the apical response. For example, a proximal event that displays a second-order Hill dose-response curve could not be a step in an apical response that exhibits a first-order Hill dose-response curve (Ong et al. 2010, Chow et al. 2011). In this way, quantitative dose-response modeling may provide some mechanistic insights into the role of various events (Simons and Chow 2012). In addition, various analytical tools can be employed to gain mechanistic insight that is available only when the Hill coefficient is equal to one (Dougherty et al. 2012, Ong et al. 2010). A Hill coefficient of two or greater may indicate involvement of transcription factors that act as dimers or higher-order multimers. Alternatively, the observation of Hill coefficients greater than one may also result from ligand-induced conformational changes in binding proteins that function as dimers or multim-

ers (Koshland 1996, Koshland and Hamadani 2002, Levitzki and Koshland 1969). Furthermore, it would be instructive to know the details of ligand binding to ERα in cell-free extracts, in whole cells and in whole animals. One would also want data on the genomic responses *in vitro* and in whole animals. In addition, these data would need to be of sufficient quality to support quantitative dose-response modeling.

Second, additional data are needed to provide dose-response information at different times for those events hypothesized to be KEs. Ideally, these data would be collected under the same experimental conditions as that for the apical event. When performed, interim sacrifices in a cancer bioassay often provide this type of data (*e.g.*, NTP 2006). Such data are necessary for constructing a time line of the KEs and providing data for the Dose-Time Concordance Table (Table 2).

Third, a decision should be made concerning the best experimental system for examining the effects of modulatory factors. For example, if ER-knockout mice are to be used, then high-quality dose-response data, as discussed above, should be collected from
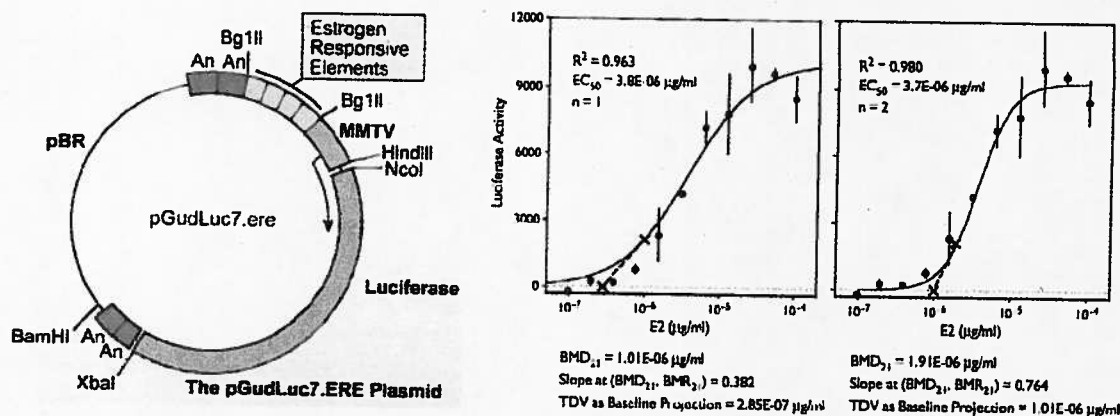
Figure 9. Details of one of the heterologous expression systems that could be used to substitute for the uterotrophic assay. Left: Stably transfected Luc reporter plasmid BG1Luc4E2 cell line from ICCVAM. Right: Concentration-response of the BG1Luc4E2 cells to estradiol showing fits to both first- and second-order Hill functions and the results of the transitional dose value calculation using the baseline projection method (Eq. 3,4 and 5). Please see Supplementary Content for another example.

both normal and knock-out mice. Alternatively, if tissue culture and high throughput studies are selected, then appropriate tissue culture lines could be used and would need to be identified.

Potential utility of understanding the MOA for uterotrophy. One potential result of the greater understanding deriving from more complete experimental data would be the potential for increased usage of in vitro assays measuring KEs and AEs as a screen to identify the chemicals to be assessed further in the uterotrophic assay, a scheme that is consistent with Tox21. The Q-KEDRF seems the best means of demonstrating this consistency. The Interagency Coordinating Committee on the Validation of Alternative Methods has validated a whole cell assay system (Figure 9; BG1Luc ER TA) to assess the activity of different test compounds. Yamasaki et al. (2002, 2003, 2004) measured the response of a reporter gene system as well as the uterotrophic response in whole animals but did not attempt to conduct IVIVE to determine the quantitative relationship between the two—both the reporter gene assay and the in vivo assay were used only for identification of biological effects.

One important aspect of uterotrophy as a model system is that it exemplifies the likely existence of thresholds in MOAs that include receptor binding as a KE. A TDV or range is located at the point where the rising portion of the dose-response begins (Murrell et al. 1998, Sand et al. 2006). Because the binding assays were conducted *in vitro* and the units of dose and routes of exposure were not consistent among the *in vivo* studies, it is difficult to draw conclusions about the numerical values of these either possible threshold values or TDVs, but the ability to estimate these values can, in some cases, provide great insight about the MOA (*e.g.*, Simon et al. 2009).

The value of the Hill coefficient can be important in determining whether linear or nonlinear extrapolation should be used for modeling various KEs or the Adverse Outcome. For the example of uterotrophy here, the ability to obtain insights from quantitative data is mitigated by the relative paucity of the data. Inspection of Figure 9 suggests that for this *in vitro* response in BG1LucE42 cells, both first and second order Hill models provide equally good fits to these data. Notides et al. (1981) did observe a shift in the Hill coefficient with increasing concentrations of ERα and attributed this increase to the

formation of homodimers with greater availability of ERα. The uterotrophic response itself is generally considered to follow a first-order Hill function but the data from Naciff et al. (2003) seem clearly second order, possibly for this reason. Additional data collection should provide greater certainty regarding the order of the Hill function.

Potential TDVs for the responses in Figures 8 were estimated using the baseline projection method of Murrell et al. (1998) and as the BMD_{21} value as noted by Sand et al. (2006; Table 6). Silkworth et al. (2005) also suggest a method for baseline projection. Details of this method are provided in the next section and in the Supplementary Content.

Alternative Dose Levels from the Hill function for ordering KEs. The Hill model is a three or four parameter equation for a nonlinear relationship between dose and response. The model was first applied by A.V. Hill in 1910 to describe the relationship between oxygen tension and saturation of hemoglobin (Hill 1910). In pharmacology and toxicology, the Hill model has been used extensively to describe the relationship between the dose of a xenobiotic and a biological response (Goutelle et al. 2008, Wagner 1968). In another very recent paper examining the shape and steepness of dose-response relationships for continuous endpoints, the Hill model and the exponential model were both found to provide adequate fits to a large number of data sets covering many continuous endpoints (Slob and Setzer 2014).

For consideration of MOA, location and steepness of the dose-response may help order the events within the dose range. One would wish to know the approximate dose at which the rising portion of the dose-response begins, in other words, the TDV.

A form of the Hill model is shown below and it will be used later to examine responses to estrogenic chemicals. We also provide in Table 7 the inverse equation for calculating the dose at a specified response, for example, the BMD, and the equation for the slope.

$$Response = g + \frac{(V_{max} - g)}{1 + 10^{n(\log_{10}(K_d) - \log_{10}(dose))}} \qquad (2)$$

where g = background response;
Vmax = maximal response or efficacy;
n = Hill coefficient (unitless); and

Table 7. Inverse equations and slope equations of dose-response models from EPA's benchmark dose software (USEPA 2012) to enable estimation of baseline projection values.

Model	Equation	Inverse	Derivative
Hill	$Response = \dfrac{1}{1+10^{n\left((\log_{10}(K_d)-\log_{10}(dose))\right)}}$	$\log_{10}(dose) = \log_{10}(K_d) - \dfrac{\log_{10}\left(\dfrac{1}{Response}-1\right)}{n}$	$Slope = \dfrac{n\,10^{n\left((\log_{10}(K_d)-\log_{10}(dose))\right)}}{dose\left(10^{n\left((\log_{10}(K_d)-\log_{10}(dose))\right)}+1\right)^2}$
Logistic	$Response = \dfrac{1}{1+e^{-(\alpha+\beta dose)}}$	$dose = \dfrac{-\alpha - \log_e\left(\dfrac{1}{Response}-1\right)}{\beta}$	$Slope = \dfrac{\beta e^{-(\alpha+\beta dose)}}{\left(1+e^{-(\alpha+\beta dose)}\right)^2}$
Log-Logistic or Dichotomous Hill	$Response = \dfrac{1}{1+e^{-(\alpha+\beta\log_e(dose))}}$	$\log_e(dose) = \dfrac{-\alpha - \log_e\left(\dfrac{1}{Response}-1\right)}{\beta}$	$Slope = \dfrac{\beta e^{-(\alpha+\beta\log_e(dose))}}{dose\left(1+e^{-(\alpha+\beta\log_e(dose))}\right)}$
Multistage (2nd order)	$Response = 1 - e^{-\beta_1 dose - \beta_2 dose^2}$	$dose = \dfrac{-\beta_1 + \sqrt{\beta_1^2 - 4\beta_2\log_e(1-Response)}}{2\beta_2}$	$Slope = e^{-\beta_1 dose - \beta_2 dose^2}(\beta_1 + 2\beta_2 dose)$
Weibull	$Response = 1 - e^{-\beta dose^\alpha}$	$\log_e(dose) = \dfrac{\log_e\left(\dfrac{-\log_e(1-Response)}{\beta}\right)}{\alpha}$	$Slope = \alpha\beta\,dose^{\alpha-1}e^{-\beta dose^\alpha}$
Exponential Model 2	$Response = \alpha e^{\beta dose}$	$dose = \dfrac{\log_e\left(\dfrac{Response}{\alpha}\right)}{\beta}$	$Slope = \alpha\beta e^{\beta dose}$
Exponential Model 3	$Response = \alpha e^{\beta dose^\delta}$	$\log_e(dose) = \log_e\left(\dfrac{\log_e\left(\dfrac{Response}{\alpha}\right)}{\beta}\right)\left(\dfrac{1}{\delta}\right)$	$Slope = \alpha\beta e^{\beta dose^{\delta-1}}e^{\beta dose^\delta}$

These values may be useful for ordering events within a hypothesized MOA. These equations are written to be easy to implement in spreadsheet software such as MS-Excel. Their use is not for development of regulatory criteria but rather exploration of hypothesized MOAs.

K_d = affinity or dose at the half-maximal response, a measure of potency (For concentrations, this parameter is often shown as EC_{50}, indicating a dose or concentration with a 50% of maximal efficacy.

In Eq. (2) and all equations following, common or base 10 logarithms are denoted by "$\log_{10}$" and natural logarithms are denoted by "$\log_e$." All the responses shown in Figure 8 were fit to Eq. (2). The third column in Table 6 shows the fitted values for K_d and n, the Hill coefficient.

Another method to obtain the TDV is that of Murrell et al. (1998). The baseline projection of the rising part of the curve is obtained by choosing two points by inspection, one above and one below the half-maximal response. The slope of the rising portion is calculated as the ratio of the differences of the dose and response values of these two points.

$$Slope = \frac{R_1 - R_2}{\log_{10}(dose_1) - \log_{10}(dose_2)} \quad (3)$$

where R_i = fractional response levels above and below 0.5.

This slope will likely be very close to that at the half-maximal response. Hence, using 0.5 as the measure of the response at the K_d value on a zero-to-one scale, the dose at the onset of the rising portion of the dose-response is calculated as:

$$TDV = \log_{10}(K_d) - \frac{0.5}{Slope} \quad (4)$$

The results are shown in column 4 of Table 6.

For the form of the Hill model shown in Eq. (2), the dose at any fractional response level, for example, 0–1, can be obtained as follows:

$$\log_{10}(dose) = \log_{10}(K_d) - \frac{\log_{10}\left(\dfrac{1}{Response}-1\right)}{n} \quad (5)$$

Equation (5) was used to calculate the BMD_{21}, identified as a TDV by Sand et al. (2006; Table 6).

Once the Hill model parameters for the dose-response (Eq. 2) have been obtained from fitting software, the results of Eqs. (3–5) can be easily obtained with spreadsheet software or even a hand calculator. Only the Hill coefficient, n, and the common logarithm of the half-maximal concentration, $\log_{10}(K_d)$, are needed.

These doses are referred to as transitional because their location marks the approximate transition to the rising portion of the dose-response (Sand et al. 2006). The method of Murrell et al. (1998) explicitly considers steepness with a calculation of the slope. The BMD_{21} is the point at which the generalized Hill model transitions to the rising phase, as indicated by higher derivatives of the model (Sand et al. 2006).

Measurements of binding to the estrogen receptor show very similar slope-based TDVs. One might expect gene expression changes to occur at a lower dose than uterine weight gain. The slope-based TDV for the increase in expression of vitamin D-dependent intestinal calcium-binding protein (Calb3) from Naciff et al. (2003) is about half than that for uterine weight gain in the same study; however, the BMD_{21} values for these two effects are much more similar (Table 6).

In contrast, the data from Heneweer et al. (2007) show about a two-fold increase in the BMD_{21} but similar slope-based TDVs. Both studies used immature female Sprague-Dawley rats so the difference in the relationship of TDVs between the two studies is likely due to the small number of data points and uncertainty in the fit. The fact that these two methods of calculating a transitional dose range/value give different results for two similar studies would be a reason to obtain further details of the biological role of Calb3 in the uterotrophic response. Highlighting the need for additional qualitative information about the biology underlying the MOA is a great benefit of the use of the Q-KEDRF.

Confidence limits could be likely determined for these TDVs, but the point of their use is to obtain evidence regarding the timing and role of events in a hypothesized MOA. The relationship between Calb3 and uterine weight is not yet known (Naciff et al. 2003, Heneweer et al. 2007). Hence, a review of the literature and possibly some laboratory studies would go further in addressing this particular data gap.

Last in the table are three measurements for increases in uterine cell proliferation, blood flow, and weight gain reported in OECD (2003). All three studies were conducted in rats and the TDVs may suggest that the order of events along the dose continuum is:

1) cell proliferation;
2) increased blood flow measured by uterine peroxidase; and,
3) uterine weight gain.

Both types of TDV for all three studies were expressed in units of μg/animal/d. Here, the slope-based TDV suggests that cell proliferation may be a low dose-response, whereas the slope-based TDVs for increases in blood flow and uterine weight gain occur fairly close to each other along the dose continuum. The TDVs as the BMD_{21} for these three responses are more challenging to interpret. The reason is likely that the slope-based TDVs used the actual data to develop a slope value and the BMD_{21} TDV uses the fitted Hill coefficient. In all three cases, the fitted Hill coefficients had low values and the fits were performed on data with six or fewer dose values.

Another example of this type of quantitative MOA analysis can be found in recent work on the MOA of dioxin liver carcinogenesis in rats (Budinsky et al. 2014, Simon et al. 2009). Both papers present figures showing dose-response plots of different events in the MOA ordered by increasing K_d values and increasing Hill coefficients.

In all likelihood, statisticians can think of much more sophisticated analyses using the slope of the dose-response. Such approaches could use expressions for the slope of the dose-response and attempt to discover in what dose ranges the most rapid change occurs. However, for the purposes of working out events within a hypothesized MOA, easily calculated values such as K_d or the TDV can be very useful.

There may be additional insight gained from using a baseline projection method similar to that obtained at the half-maximal response level using the procedure of Murrell et al. (1998). Table 7 provides equations for commonly used empirical dose-response models, the corresponding inverse equations that solve for dose as the independent variable based on a chosen response, and equations for the dose-response slope at any point. In some instances, these equations can be used to project to the baseline or zero response using the slope at the chosen level of response (Figure 9; Supplementary Content). The inverse equations in Table 7 simply express the dose corresponding to a chosen fractional response (assuming "1" is the maximal response). Using these equations should prove simpler than obtaining an implicit solution. The slope equations in Table 7 provide a means of calculating the slope at the benchmark point (BMD, BMR).

Baseline projection from the 21% response level is shown graphically in Figure 9. Although the values for the EC_{50} are very close, the BMD_{21} values differ by a factor of 2 and the baseline projections from the 21% response level differ by over three-fold. An examination of these differences may help discover the sequence of KEs in a proposed MOA.

As noted, the Supplementary Content provides another example calculation of this baseline projection method that incorporates both the location and steepness of the dose-response at a chosen point and how to use such information in thinking about a hypothesized MOA.

Comparing the values of the Hill coefficients of various events in a hypothesized MOA may provide additional insight and contribute to the decision of whether to assume the adverse outcome follows a linear or nonlinear MOA. Ligand binding and the constellation of early steps in gene transcription may have Hill coefficients close to unity and thus their dose-response might be considered linear (Murrell et al. 1998, Budinsky et al. 2014). KEs that have Hill coefficients with values of 2 or greater invariably indicate the MOA for the adverse outcome will be nonlinear (Chow et al. 2011).

Log-steepness, measured by the ratio of the BMD_{10} to the BMD_{05}, was considered for use in ordering events with a hypothesized MOA (Slob and Setzer 2014). The dose-response data provided in EPA (2005c) was used to obtain values of log-steepness for KEs in the MOA of cacodylic acid (Tables 2 and 3; Figure 3). The three KEs are cytotoxicity, proliferation, and hyperplasia occurring at 10 weeks (Table 3). Appendix D of this EPA publication contains the BMDS output for these three KEs. The values for log-steepness calculated as the BMD ratio for these three KEs (cytotoxicity, proliferation, and hyperplasia) were 2.1, 1.1, and 1.4, respectively. Slob and Setzer (2014) note that log-steepness estimated as the BMD ratio is imprecise, and, while this is only a single example, this easily calculated value did not prove helpful in ordering KEs within a hypothesized MOA. Further work is needed to determine whether this measure of log-steepness can indeed help inform details of MOA.

Constructing a Dose-Time Concordance Table may also help to identify late occurring KEs. These late KEs in the modes of action of complex adverse outcomes such as cancer or developmental effects, may be highly nonlinear and will likely have high-valued Hill coefficients (Brown et al. 2012, Hanahan and Weinberg 2000, 2011, Simon et al.

2009). In some cases, sufficient information about the MOA will be available to select some KEs to use as appropriate precursors to the adverse outcome such as was done by EPA for dimethylarsinic acid. The ability to select appropriate precursor KEs will require quantitative knowledge of the relationship between that KE and the adverse outcome. When the knowledge is available, such precursor events can be used as the basis for risk assessment (Simon et al. 2009, USEPA 2005a, Thompson et al. 2014).

Application of knowledge of the MOA for uterotrophy in risk assessment. A number of host, life stage, and environmental factors likely will modulate human responses to chemicals shown to be estrogenic in the uterotrophic assay and in surrogate in vitro assays. Because many potentially estrogenic chemicals contain one or more hydroxyl groups that interact with specific ligand-binding pockets in ERα, the metabolism of these chemicals in the enterocytes lining the gastrointestinal tract and the liver may result in their inactivation. Hence, for some chemicals, first pass serves as a detoxification process.

For example, bisphenol A (BPA) is almost completely inactivated by phase II metabolism in enterocytes and liver by both glucuronidation and sulfation. These processes occur in both humans and rats (Hengstler et al. 2011). Differences in glucuronidation and sulfation of BPA in rats and humans exist and may provide the basis for interspecies extrapolation of metabolism and consequent bioavailability of BPA (Mazur et al. 2010). Alternatively, these data may be used to improve PBPK models of BPA (Fisher et al. 2011, Teeguarden et al. 2005).

Modulating factors for estrogenic responses in humans. After oral ingestion, it is not possible to detect free BPA in plasma in adult humans (Willhite et al. 2008). PBPK modeling suggests that levels of free BPA in very young children may be higher than in adults due to lower glucuronidation capacity during the first 2 months of life (Edginton and Ritter 2009, Mielke and Gundert-Remy 2009). Free BPA has been detected in the urine of premature infants in neonatal intensive care and its source may be medical devices and the need to deliver medical interventions directly via the blood (Calafat et al. 2009). In contrast, free BPA has not been detected in the urine of full-term healthy infants up to 44 days in age (Nachman et al. 2013). This fact suggests that the glucuronidation capacity in healthy infants is sufficient to metabolize BPA from environmental exposures.

Polymorphisms in uridine 5'-diphospho-glucuronosyltransferase enzymes that conjugate glucuronide may be a potential ModF (Allegaert et al. 2009, Court 2010, Girard et al. 2007, Guillemette et al. 2010, Krekels et al. 2012, Mercke Odeberg et al. 2006, Miyagi and Collier 2011, Strassburg et al. 1997, de Wildt et al. 1999). As noted, differences in glucuronidation occur with gender and age. Diet may also be a factor in the ability to inactivate estrogenic chemicals (Navarro et al. 2009, 2011, Saracino et al. 2009). In all cases of oral exposure, the actual exposure needs to be considered in a quantitative fashion—the inability to detect free BPA in the urine of normal infants suggests that exposures may be sufficiently low that glucuronidation is essentially complete (*e.g.*, Ye et al. 2012). There may be exposures to estrogenic chemicals by routes other than oral, for example, dermal or inhalation, for which glucuronidation does not occur. However, these exposures appear to be miniscule (Geens et al. 2012).

The occurrence of male reproductive tract pathologies in offspring of women administered diethylstilbestrol (DES) during pregnancy suggests that both a lowest-observed-adverse-effect level and a NOAEL exist for these developmental effects. Because no formal clinical trials had been conducted with DES, the total dose varied among clinics by an order of magnitude or more. Male reproductive tract abnormalities were observed in offspring of mothers receiving higher total doses of DES, that is, 12–18 g during pregnancy (Dietrich 2010, Golden et al. 1998), whereas no clear increase was observed in reproductive tract effects in offspring of mothers administered 1.4 g of DES during pregnancy (Leary et al. 1984).

Exposure to more than one estrogenic chemical, such as dietary phytoestrogens, may interact with, or complement, endogenous or other exogenous chemicals. As noted, at sufficient doses, estrogenic chemicals act as anti-androgens in males. However, dose addition of these chemicals is unlikely unless at least two of the doses occur in the rising portion of the dose–response curve (Borgert et al. 2012). Quantitative aspects of dose-response such as affinity, efficacy, and potency need to be considered for chemicals that act via receptor binding—simply using dose addition and some measure of relative potency will be inadequate for risk assessment (Borgert et al. 2012).

The examination of the MOA for uterotrophy requires *in vivo* measurement of the adverse outcome/apical endpoint and includes *in vitro* measurements of the MIE, genomic data, and physiological measures of KEs. Hence, this example demonstrates the use of data from tiers 1–4 of the toxicity resource pyramid of the RISK21 Roadmap (Figure 1), and illustrates the strength of MOA analyses in terms of generating data useful for risk assessment purposes.

Discussion

The MOA/HRF along with the Q-KEDRF described here provides a strong foundation for using the information gathered as a means of reducing uncertainty in risk assessments. The KEDRF laid out the approach for harnessing the extensive available data for the KEs within a putative MOA. The Q-KEDRF provides additional tools with which to gain further insights about how the KEs relate to each other and to the adverse outcome/apical event in a quantitative way in both the dose- and time-dimensions.

In risk assessment, the greatest quantitative impact comes from the choice of a linear approach versus a nonlinear approach for modeling the dose-response for the critical effect or apical effect of concern. The dose-responses for the KEs can be used to inform the shape of the dose-response for the apical effect of concern. For receptor-mediated effects, as noted, quantitative dose-response modeling can provide much greater understanding. For example, if the dose-responses of some or all KEs exhibit biological thresholds, for example, cytotoxicity of the liver and kidney induced by chloroform (Andersen et al. 2000), then the combination of events will also display a dose threshold. Alternatively, if the dose-responses for KEs do not exhibit dose thresholds, then the combination of events may result in a linear dose-response for the apical event. The ability to calculate possible threshold or transition dose values from quantitative dose-response modeling provides a means to determine whether linear or nonlinear extrapolation is appropriate (Table 7).

It is increasingly clear that account has to be taken of those ModFs that could influence the shape of the dose–response curve, the efficacy or magnitude of the apical response or selected critical effect, and the potency or location along the dose continuum. For example, how much variation can be expected for a particular ModF? Again, this depends on the underlying biology. Sufficient variation may "linearize" the dose-response of the apical event (Conolly et al. 2005, Lutz 2001). The question then is: will this amount of variation "linearize" the population dose-response to a sufficient extent to support the choice of linear low-dose extrapolation? As a generalization, ModFs that are likely to modify the dose-response characterization as part of the risk assessment process will be relatively frequent in the population (given that dose-response is a population feature). Some of these ModFs are "inevitable" and are characteristics of the general population (sex, age, and genotype); others are "manageable" and are characteristic of specific subpopulations (smoking, diet, and weight). Additional research on this topic and the overall role of ModFs is essential to inform the consideration of ModFs and their effect on MOA as part of problem formulation.

At this point in the history of risk assessment, the utility of the Q-KEDRF remains to be determined: experience in conducting real-world risk assessments will demonstrate any value added. Certainly, the Dose-Time Concordance table and Dose-Response Species Concordance table for KEs and ModFs (Tables 2–4) should provide a significant amount of help. The National Research Council recently reviewed EPA's Formaldehyde risk assessment and as part of that review, suggested that the documentation for chemical-specific risk assessments in the IRIS program be organized around informative tables (NRC 2011). The Dose-Time and Dose-Response Species Concordance tables could be very useful in that effort.

At present, the full utility of the Q-KEDRF has barely begun to be realized. The example of rat uterotrophy, while being arguably the best documented physiological response to the extensively studied steroid hormones, clearly demonstrates not only the shortcomings in the available data but also how much actual insight can be acquired through the development of a Q-KEDRF for a specific response. The Q-KEDRF will likely change as experience in using it is gained. Nonetheless, some of the basic issues discussed here will likely become hallmarks of any framework implemented to understand the MOA of a particular adverse outcome. These issues include: (1) separating KEs from putative KEs and (2) understanding the relationship between KEs based upon their dose-response and the timing of their occurrence. Use of this information can significantly improve risk assessments by reducing uncertainty and fostering the incorporation of this information into easy-to-use tables.

Acknowledgments

Declaration of interest

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Supplementary material available online

Supplementary Content to be found online at http://informahealthcare.com/doi/abs/10.3109/10408444.2014.931925.

From:	Philpott, Olivia
Sent:	Wednesday, August 13, 2014 2:28 PM
To:	Powers, Christina
Subject:	RE: Reservation Request: B249 on Aug. 20th (Arsenic IRIS)

Christy,

B249 is not available, I have your scheduled in B230. Is this a video, how many people? You can do slides in B230 using the Tandberg. If not, we have a portable projector for usage in B230.

Regards

Olivia Philpott
Information Management Technician / NCBA
National Center for Environmental Assessment
Research Triangle Park Division
U.S. Protection Agency
Research Triangle Park, NC 27711
Phone: (919) 541-4915

From: Powers, Christina
Sent: Wednesday, August 13, 2014 1:53 PM
To: Philpott, Olivia
Cc: Powers, Christina
Subject: Reservation Request: B249 on Aug. 20th (Arsenic IRIS)

Hi Olivia,

Would it be possible to reserve Room B249 on Wednesday, Aug. 20th from 1-2 p.m.? I need to meet with members of the arsenic team and we need to have access to a projector / screen but B349 is full.

Any assistance you can offer would be greatly appreciated.
Thanks!
Christy

Christy Powers
Biologist
National Center for Environmental Assessment (B 220-I)
Office of Research and Development
U.S. Environmental Protection Agency
Ann Arbor, MI

Tel: 734.214.4243
E-mail: powers.christina@epa.gov

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Thanks!
Christy

Christy Powers
Biologist
National Center for Environmental Assessment (B 220-I)
Office of Research and Development
U.S. Environmental Protection Agency
Ann Arbor, MI

Tel: 734.214.4243
E-mail: powers.christina@epa.gov

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Subject:	Meeting Forward Notification: iAs AOP Team Planning
Location:	RTP-B349-Max40/RTP-Bldg-B
Start:	Tue 8/12/2014 1:00 PM
End:	Tue 8/12/2014 2:00 PM
Recurrence:	(none)
Organizer:	Powers, Christina

Your meeting was forwarded

Powers, Christina has forwarded your meeting request to additional recipients.

Meeting
iAs AOP Team Planning

Meeting Time
Tuesday, August 12, 2014 1:00 PM-2:00 PM.

Recipients
Gift, Jeff

All times listed are in the following time zone: (UTC-05:00) Eastern Time (US & Canada)

Sent by Microsoft Exchange Server 2013

1469

REVIEW ARTICLE

The use of mode of action information in risk assessment: Quantitative key events/dose-response framework for modeling the dose-response for key events

Ted W. Simon[1], S. Stoney Simons Jr.[2], R. Julian Preston[3], Alan R. Boobis[4], Samuel M. Cohen[5], Nancy G. Doerrer[6], Penelope A. Fenner-Crisp[7], Tami S. McMullin[8], Charlene A. McQueen[9], J. Craig Rowlands[10], and RISK21 Dose-Response Subteam[11]

[1]Ted Simon LLC, Winston, GA, USA, [2]Steroid Hormones Section, NIDDK/LERB, National Institutes of Health, Bethesda, MD, USA, [3]U.S. EPA/National Health and Environmental Effects Research Laboratory, Research Triangle Park, NC, USA, [4]Imperial College London, London, UK, [5]University of Nebraska Medical Center, Omaha, NE, USA, [6]ILSI Health and Environmental Sciences Institute, Washington, DC, USA, [7]Consultant, North Garden, VA, USA, [8]Dow Corning, Midland, MI, USA, [9]U.S. EPA/National Health and Environmental Effects Research Laboratory, Research Triangle Park, NC, USA, and [10]The Dow Chemical Company, Toxicology and Environmental Research & Consulting, Midland, MI, USA, [11]ILSI Health and Environmental Sciences Institute, Washington, DC, USA.

Abstract

The HESI RISK21 project formed the Dose-Response/Mode-of-Action Subteam to develop strategies for using all available data (*in vitro, in vivo*, and *in silico*) to advance the next-generation of chemical risk assessments. A goal of the Subteam is to enhance the existing Mode of Action/Human Relevance Framework and Key Events/Dose Response Framework (KEDRF) to make the best use of quantitative dose-response and timing information for Key Events (KEs). The resulting Quantitative Key Events/Dose-Response Framework (Q-KEDRF) provides a structured quantitative approach for systematic examination of the dose-response and timing of KEs resulting from a dose of a bioactive agent that causes a potential adverse outcome. Two concepts are described as aids to increasing the understanding of mode of action—Associative Events and Modulating Factors. These concepts are illustrated in two case studies; 1) cholinesterase inhibition by the pesticide chlorpyrifos, which illustrates the necessity of considering quantitative dose-response information when assessing the effect of a Modulating Factor, that is, enzyme polymorphisms in humans, and 2) estrogen-induced uterotrophic responses in rodents, which demonstrate how quantitative dose-response modeling for KE, the understanding of temporal relationships between KEs and a counterfactual examination of hypothesized KEs can determine whether they are Associative Events or true KEs.

Abbreviations: *AChE* acetyl cholinesterase, *AE* associative event, *AOP* adverse outcome pathway, *As3mt* arsenic methyltransferase, *AUC* area under the curve, *BMDL* benchmark dose lower confidence limit, *BMR* benchmark response, *BPA* bisphenol A, *BrdU* bromodeoxyuridine, *BuChE* butyrylcholinesterase, *ChE* cholinesterase, *CPF* chlorpyrifos, *CYP450* cytochrome P450, *DES* diethylstilbestrol, *DMA^III* dimethylarsinic acid (reactive metabolite trivalent), *DMA^V* dimethylarsinic acid, *DMPS* dimercaptopropanesulfonic acid, *DR* dose-response, *EC* European Commission, *EC_{50}* median effective concentration, *EFSA* European Food Safety Authority, *EPA* Environmental Protection Agency (US), *ER* estrogen receptor, *ERα* estrogen receptor alpha, *HESI* Health and Environmental Sciences Institute, *HRF* Human Relevance Framework, *ILSI* International Life Sciences Institute, *IVIVE* in vitro to in vivo extrapolation, *KE* key event, *KEDRF* Key Events/Dose-Response Framework, *L-NAME* L-NG-nitroarginine methyl ester, *ModF* modulating factor, *MIE* molecular initiating event, *MOA* mode of action, *MOE* margin of exposure, *NOAEL* no-observed-adverse-effect level, *NRC* National Research Council, *OECD* Organisation for Economic Co-operation and Development, *OP* organophosphate, *PBPK* physiologically based pharmacokinetic, *PD* pharmacodynamic, *PON1* Paraoxonase 1, *PR* progesterone receptors, *Q-KEDRF* Quantitative Key Events/Dose-Response Framework, *QSAR* quantitative structure-activity relationship, *RBC* red blood cell, *REACH* Registration, Evaluation, Authorisation and Restriction of Chemicals, *RIP140* receptor interacting protein 140, *SAM* S-adenosyl methionine, *SRC-1* steroid receptor coactivator-1, *TCPy* 3,5,6-trichloro-2-pyridinol, *TDV* traditional dose value, *WoE* weight of evidence.

Address for correspondence: Nancy G. Doerrer, ILSI Health and Environmental Sciences Institute, 1156 15th Street, NW, Washington, DC 20005, USA. Tel: +202-659-3306. E-mail: ndoerrer@hesiglobal. org; URL: http://www.risk21.org

Table of Contents

Introduction

As society progresses through the second decade of the 21st century, there is increased pressure to embrace new ideas and new information in the practice of toxicology and risk assessment. Modern biological science has provided many assessment tools—genomics, transcriptomics, proteomics, metabolomics, and others—that enable scientists to dissect and ultimately understand the biological pathways underlying toxicity. Disruption of these pathways is associated with adverse outcomes.

The progression of this understanding of these adverse outcome pathways fosters and enables the use of these new tools in the practice of chemical risk assessment (Ankley et al. 2010, NRC 2007). What is needed is the knowledge of the biological pathways that underlie a given toxicity and an estimate of the degree or amount of disruption each pathway can tolerate without the occurrence of pathway-specific toxicity (Boekelheide and Andersen 2010, Boekelheide and Campion 2010, Hartung and McBride 2011). The use of mode of action (MOA) currently is the most reliable way for developing sufficient knowledge and understanding of these biological pathways.

RISK21 project

For a number of years, the International Life Sciences Institute (ILSI) Research Foundation has assembled cross-disciplinary working groups to examine current risk assessment approaches for evaluating dose-response and identifying safe exposure levels (Julien et al. 2009). Recently, these efforts were applied to four categories of bioactive agents—food allergens, nutrients, pathogenic microorganisms, and environmental chemicals—and from the lessons learned, a common analytical framework was developed for understanding MOA—the Key Events/Dose-Response Framework (KEDRF; Boobis

et al. 2009, Buchanan et al. 2009, Julien et al. 2009, Ross et al. 2009, Taylor et al. 2009).

The present paper describes ways to incorporate information about the timing of occurrence and quantitative dose-response of Key Events (KE) into the KEDRF. This expanded framework is known as the Quantitative Key Events/Dose-Response Framework or Q-KEDRF. In one sense, this is a "how-to" paper, which describes methods to incorporate additional information for understanding the particulars of the MOA of a chemical. In addition to a discussion of these methods, examples are provided for illustration.

Dose-response/Mode-of-Action Subteam

A central issue in 21st century toxicology and risk assessment is dose-response analysis and its extrapolation to human exposure levels. Building on the KEDRF, the Dose-Response (DR)/Mode-of-Action (MOA) Subteam within the ILSI Health and Environmental Sciences Institute's (HESI's) RISK21 project was formed to develop a clear strategy for using all available data (*in vitro, in vivo,* and *in silico*) in both qualitative and quantitative ways to develop the methods to be used in next-generation risk assessments of substances. The gathering of these various types of data is best accomplished in a tiered fashion suggested by the red triangle labeled as "Toxicity" in the upper left portion of Figure 1.

The DR/MOA Subteam has three main objectives: 1) to provide a forum to discuss approaches to dose extrapolation in human health risk assessment; 2) to address how an understanding of MOA will influence low-dose extrapolation; and 3) to enhance the existing MOA/Human Relevance Framework (HRF) and KEDRF. Specifically, this third objective aims to use quantitative dose-response and temporal information about both KEs and the adverse outcome in a more robust way. Consistent with all HESI projects, participation in the Risk21
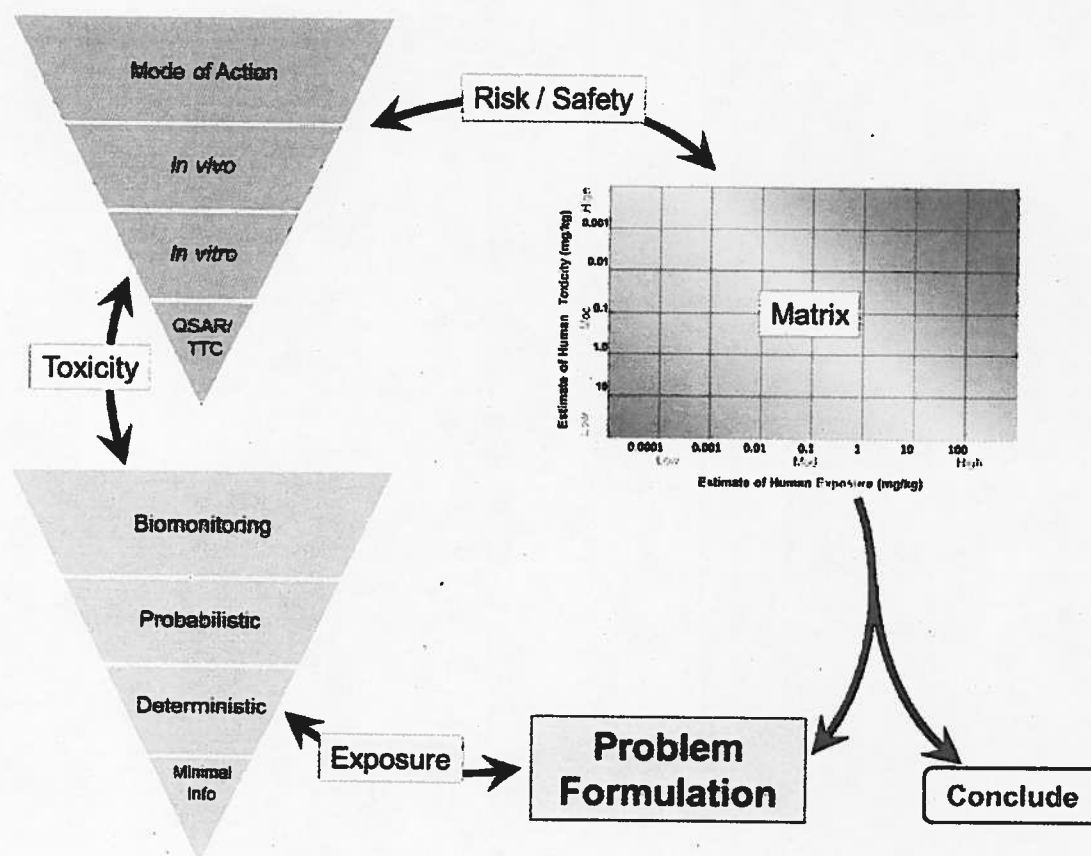
Figure 1. The HESI RISK21 Roadmap and Matrix.

Dose-Response Subteam included tripartite representation from government, academia, and industry, with subteam co-leadership provided by expert scientists from academia and industry.

History and uses of MOA/HRF frameworks

MOA is defined specifically in the US Environmental Protection Agency's (EPA's) 2005 *Guidelines for Carcinogen Risk Assessment* as follows:

> ... *a sequence of Key Events and processes, starting with interaction of an agent with a cell, proceeding through operational and anatomical changes, and resulting in cancer formation. A "key event" is an empirically observable precursor step that is itself a necessary element of the mode of action or is a biologically based marker for such an element. Mode of action is contrasted with "mechanism of action," which implies a more detailed understanding and description of events* (USEPA 2005a).

While necessary, single KEs by themselves are not usually sufficient for the adverse outcome to occur, as noted by Julien et al. (2009):

> *Hence, a key event is a necessary, though not a sufficient, step in a process that results in a specific adverse effect.*

Julienetal.(2009)alsoprovidesomehistoricalperspectiveontheconcept of MOA and broadened the definition as the "fundamental biological events and processes that underlie the effect of a bioactive agent". In risk assessment, consideration of MOA likely originated from the work of Lehman-McKeemanetal.(1989)onmaleratnephrotoxicityassociated with accumulation of alpha 2μ-globulin, the work of Cohen and Ell-wein (1990) and Cohen (1995) on bladder carcinogenesis, and that of Faustman et al. (1997) on the evaluation of mechanisms of developmental toxicity.

The KEDRF provides a structured approach for systematic examination of KEs that occur between the initial dose of a bioactive agent and the final or apical effect of concern (Julien et al. 2009). Here, not only are the timing of KEs and the quantitative aspects of dose-response examined, but also two additional concepts for understanding MOA are discussed—Associative Events (AEs) and Modulating Factors (ModFs). These concepts were defined in Andersen et al. (2014). AEs essentially provide biomarkers for KEs, and a full definition is provided in a later section. ModFs affect the timing and/or dose-response of KEs and include variability in homeostasis or repair capacities, adaptive or immune mechanisms, enzyme polymorphisms, and other biological factors. The nature and strength of ModFs varies between individuals and in the same individual over time. Life stage, disease state, genetics, lifestyle, and other factors underlie this inter- and intra-individual variability. The Q-KEDRF provides a means to incorporate ModFs in specific situations (described below), and thus, to understand how these result in distributions of population sensitivity in the dose-response of the various KEs and, ultimately, the adverse outcome.

MOA included in regulatory guidance

Government regulatory agencies around the world have incorporated MOA/HRFs into guidance documents because of their ability to inform risk assessments. For example, the European Commission (EC) has incorporated MOA in its risk assessment guidance for industrial chemicals and biocides, and the US EPA's *Guidelines for Carcinogen Risk Assessment* specifically emphasizes the use of MOA information for interpreting and quantifying the potential cancer risks to humans (EC-JRC 2003, USEPA 2005a). In addition, EPA's *Supplemental Guidance for Assessing Susceptibility from Early-Life Exposure to Carcinogens* (or Supplemental Guidance) also relies on knowledge of the MOA (USEPA 2005b). The EPA has also drafted a *Framework for Determining a Mutagenic Mode of Action for Carcinogenicity* that is also based upon MOA, but this guidance has not yet been finalized (USEPA 2007). Health Canada considers MOA in development of drinking water guidelines and pesticide resistance management labeling (Health Canada 1999, 2009, 2011, Liteplo and Meek 2003).

The European Food Safety Authority (EFSA) includes a MOA assessment in its guidance on Harmonizing Cancer and Non-cancer Risk Assessment Approaches (EFSA 2005). MOA is recommended in the EC Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH) Regulation guidance for conducting a chemical safety assessment, and in the new "classification, labelling, and packaging" regulation on chemical substances and mixtures (EC 2008). The Organisation for Economic Co-operation and Development (OECD) recommends using MOA to support the building of chemical categories or when using read-across approaches (http://www.oecd.org/chemicalsafety/risk-assessment/groupingofchemicalschemicalcategoriesandread-across.htm). OECD has further embraced the concept of MOA in its recent use of adverse outcome pathways (AOPs; Ankley et al. 2010, OECD 2013). With the push to use more systematic and weight-of-evidence (WoF) approaches in risk assessment, both the recognition of the value and importance of the MOA/HRF and KEDRF and their use in risk assessments will increase.

MOA reduces uncertainty and informs quantitative risk assessment

MOA is a fundamental component of risk assessment for the classification of carcinogens and systemic toxicants, and informing the choice of whether a nonlinear or linear approach to low-dose extrapolation is appropriate. Evaluators can use quantitative kinetic and/or dynamic data considered in MOA analysis in at least five ways. These are listed below, along with specific examples:

1) replace default species extrapolation factors;
2) evaluate more directly the relevant concentrations in the target tissue;
3) determine the most representative dose metric;
4) choose the most appropriate quantitative dose-response model; and
5) assess quantitatively the overall relevance to humans.

Replacement of the default toxicodynamic component of the species extrapolation factor was based on species-dependent differences in the dose-response for AHR activation between

humans and rodents in a risk assessment for dioxin based on the 2006 NTP cancer bioassay (Budinsky et al. 2014, NTP 2006, Simon et al. 2009). The understanding gained by investigation into the MOA of small intestinal carcinogenesis by hexavalent chromium led to the identification of the flux of hexavalent chromium entering each segment of the small intestine as the best measure of concentration affecting the target tissue (Kirman et al. 2012, Thompson et al. 2014). The extensive work on the MOA of the pesticide chlorpyrifos (discussed in detail below) enabled the recent identification of brain cholinesterase inhibition as the most appropriate dose metric for a risk assessment based on cholinesterase inhibition (Reiss et al. 2012). An examination of the MOA of acrylamide-induced mammary tumors in F344 rats suggested that nonlinear low-dose extrapolation was a more appropriate method than linear extrapolation (Maier et al. 2012). Last, the Q-KEDRF is part of the MOA/human relevance framework (MOA/HRF) and the purpose of this larger framework is the assessment of human relevance (Boobis et al. 2006, Boobis et al. 2008, Cohen et al. 2003, Cohen et al. 2004, Cohen and Arnold 2011, Meek et al. 2003, Meek 2008, Seed et al. 2005, Meek et al. 2014a, Meek et al. 2014b).

An understanding of MOA is also needed to account for the role of metabolism in various tissues and to decide which early metabolic changes may be KEs. This understanding enables the evaluator to account for induction or inhibition of metabolism of a particular chemical and for potential first-pass effects that may increase or decrease toxicity due to metabolite formation or reduction in the systemic dose of the parent compound. Variations in patterns of toxicity with different metabolic profiles exist across species, strains and sexes in animals and across potentially susceptible subgroups and different life stages in humans. These variations need to be considered so that appropriate and defensible quantitative adjustments can be made for purposes of incorporation of these differences into risk assessments. The overall result is that MOA information can reduce uncertainties in risk assessments in a number of areas.

MOA is the foundation of 21st century toxicology testing and risk assessment

The interpretation of traditional animal toxicity studies for their relevance to humans is difficult, at times impossible, and, more often than not, fraught with controversy (Seok et al. 2013, Beyer et al. 2011, Gori 2013, NRC 1983). These studies generally use high doses resulting in considerable uncertainty when attempting to extrapolate the effects observed in animals to humans, especially when humans are experiencing much lower environmental exposures (NRC 1983). Aspects of this interpretation no less important than human relevance include: 1) the advances in understanding MOA, including the molecular and cellular events responsible for toxicity; 2) the desire to refine, reduce and replace the use of animals in regulatory toxicity testing; and 3) the need for toxicity evaluations for the large number of chemicals in commercial use. In response to these issues, the National Research Council (NRC) developed recommendations on toxicity testing that incorporated new *in vitro* and *in silico* technologies and computational systems biology to complement, and eventually replace, whole animal testing. The new strategy was presented in a report titled

Toxicity Testing in the 21st Century: a Vision and a Strategy (NRC 2007).

The report emphasized the importance of relating events leading to toxicity in the context of perturbations in biologic functions, some of which may be reversible or may represent biologically appropriate adaptations to stressors. Twenty–first century risk assessment uses the knowledge of MOA to link together perturbations in biological pathways observed in humans, in animals, in experiments with *in vitro* systems, and even those predicted by quantitative structure–activity relationships (QSAR) or other computational methods with the goal of determining the likelihood of adverse health outcomes in humans (upper left box in Figure 2).

One vital aspect of this new strategy and the vision of 21st century risk assessment is the development of appropriate prediction models (Adeleye et al. 2014, Judson et al. 2014, Patlewicz et al. 2013). Statistical approaches that attempt to correlate high throughput assay results with adverse outcomes appear to possess a level of predictivity no better than that derived from chemical structure (Thomas et al. 2012). The realization of this difficulty has fostered the curation of AOPs for use in prediction models (Landesmann et al. 2013, OECD 2013, Vinken 2013). In addition, attempts are being made to develop broad categories of MOAs for the purpose of exploiting extant knowledge across categories in a new application of read across (Briggs et al. 2012, Thomas et al. 2013, Vink et al. 2010). Understanding MOA seems to be a necessary part of eventual use of AOPs for risk assessment. Both dose and time contribute to the development of a biologically adverse response—hence, knowledge of MOA requires a detailed understanding of the dose- and time-dependency of the steps that lead from the initial interaction with a chemical to a specific toxic effect (Rowlands et al. 2014).

The Q-KEDRF—a tool for understanding MOA

MOA provides a link between exposure and the risk of adverse health outcomes—but only when the observed pathway perturbations can be characterized in terms of KEs. An important aspect of the definition of a KE is that its occurrence is necessary for the apical event. The other part of the definition is that a KE is "empirically observable." Necessity, as part of the definition, allows one to develop a counterfactual experiment for a putative KE (Figure 2, Box B2) and actually pose the question of whether it truly is a KE—if the event does not occur, will the adverse outcome occur?

Organizing questions and a toolbox for the Q-KEDRF

Box 1 provides a set of organizing questions for MOA as a prelude to applying the Q-KEDRF for specific MOA analyses. These general questions were developed from the charge questions provided to three expert panels in a workshop held at NIEHS to evaluate nuclear receptor-mediated MOAs for liver carcinogenicity (Budinsky et al. 2014, Corton et al. 2014, Andersen et al. 2014, Elcombe et al. 2014). The questions are sorted into three general areas, but in practice, there will likely be considerable overlap between the questions. Attempting to answer these questions will provide anyone engaged in MOA analysis with an understanding of the extent of knowledge.

Box 2 provides three overall categories of schemes for concise organization of the MOA information resulting from tackling the questions in Box 1. Examples of these methods are given from the papers resulting from the nuclear receptor workshop (Budinsky et al. 2014, Corton et al. 2014, Andersen et al. 2014, Elcombe et al. 2014). Necessarily, the graphical techniques, save for the flow chart, will be quantitative. Although not mentioned specifically
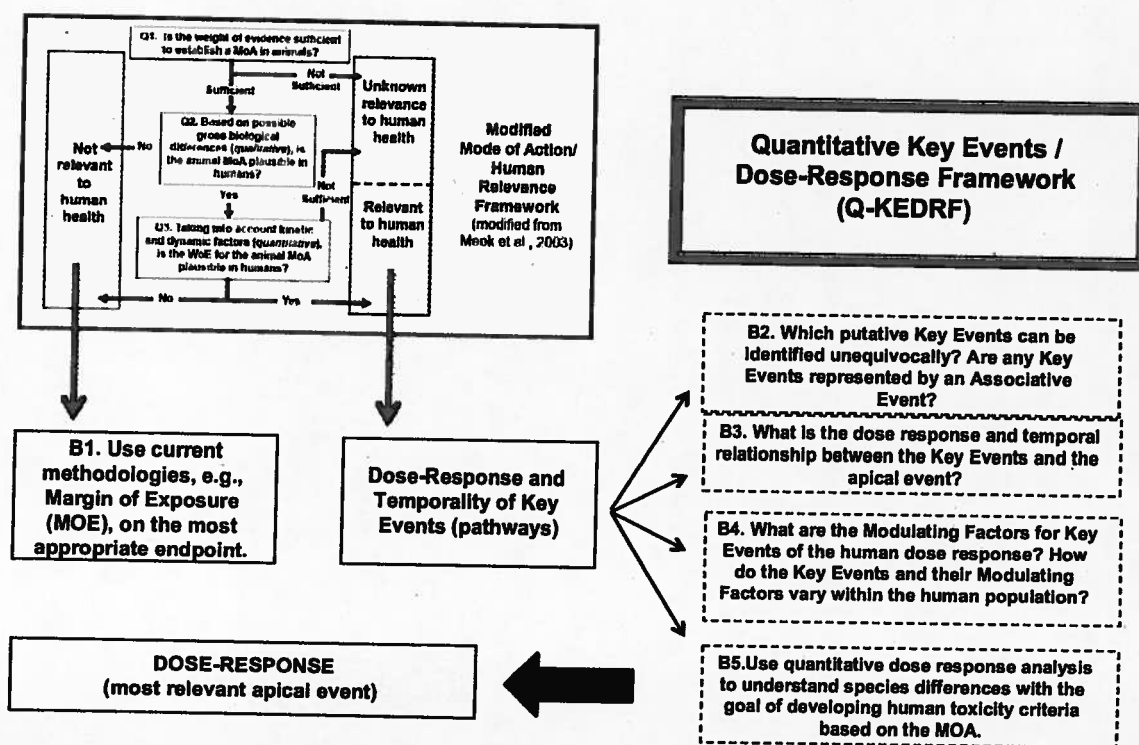

Figure 2. Quantitative Key Events/Dose-Response Framework (Q-KEDRF) and Its Relationship to the Mode of Action/Human Relevance Framework.

Box 1. Organizing questions for mode of action analysis.

Organizing Questions for MOA Consideration
- What is the proposed MOA to be evaluated by the IPCS Human Relevance Framework and modified Bradford Hill considerations?
- Which events are necessary and thus truly key events (KEs)?
- Which events are associative events (AEs)?
- What are the modulating factors (ModFs)?
- Is the proposed MOA likely to be relevant to humans?

Organizing Questions for Quantitative Dose-Response Consideration
- Are extant data sufficient for establishing dose-response relationships for proposed KEs?
- Are extant data sufficient for dose-response modeling of proposed KEs?
- What are the data gaps?
- Does the current understanding support a threshold or non-threshold DR and low dose extrapolation approach?
- On either theoretical or practical grounds, is there a dose or area-under-the-curve (AUC) level insufficient for one or more KEs or the adverse outcome (AO) to occur?

Organizing Questions for Using MOA in Risk Assessment
- Does the weight-of-evidence suggest an appropriate model or approach for the dose-response assessment?
- If so, what are the key data gaps?
- Using a value-of-information (VOI) approach (NRC, 2009; Meek et al., 2014a, b), what data would have the highest value?

in Box 2, exposure–response arrays used in the Toxicological Profiles from the Agency for Toxic Substances and Disease Registry (ATSDR) and the newer Toxicological Reviews from EPA's IRIS program could be organized around proposed KEs within one or more hypothesized MOAs.

WoE considerations for identifying key events and understanding their role in the MOA

Here, we build on the work of Julien et al. (2009) and Andersen et al. (2014) to develop the Q-KEDRF. The following definitions are used in the Q-KEDRF:

Box 2. Overview of the Q-KEDRF toolbox.

Tabular Methods
- Application Scheme for IPCS Human Relevance Framework (Figure 1 in Andersen et al., 2014)
- Comparison of Proposed MOAs (Table 4 in Corton et al., (2014))
- Qualitative Species Concordance Table (Table 4 in Elcombe et al., (2014)
- Qualitative MOA Concordance across Chemicals (Table 5 in Corton et al. (2014)

Graphical Methods
- Flow chart of each proposed MOA (Figure 7; Figure 2 in Corton et al. 2014; Figure 2 in Budinsky et al., 2014)
- Dose-Response Arrays (Figure 8)
- Quantitative Species Concordance Table (Table 3; Table 5 in Budinsky et al., 2014)
- 3D Plotting for Visualizing KEs in Dose and Time (Figure 8 in Budinsky et al., 2014; Figure 6 in Corton et al., 2014)

Quantitative/Computational Methods
- Dose-Response Modeling (BMDS, Graphpad Prism, Other tools)
- Use of Dose Surrogates (AUC, Enzyme Induction levels, etc.)
- Dose-Response Slope Analysis (Tables 6 and 7 here)

- Key Event (KE): An empirically observable causal precursor step to the adverse outcome that is itself a necessary element of the MOA. KEs are necessary but usually not sufficient for the adverse outcome in the absence of other KEs.
- Associative Events (AEs): Biological processes that by themselves are not KEs in the hypothesized MOA but may serve as reliable indicators or biomarkers for KEs. AEs can be used as surrogates or biomarkers for a KE in a MOA evaluation; depending upon the nature of the biomarker, AEs may reflect exposure to a xenobiotic, the resulting effect, or both.
- Modulating Factors (ModFs): Biological and individual factors, including control mechanisms or host factors, that can modulate the dose–response relationship of one or more KEs, thus altering the probability or magnitude of the adverse outcome (Figure 2, Box B4).

AEs can easily be thought of as biomarkers. In this regard, their relationship to KEs may need to be explored, especially if the AE is needed to measure the KE (IOM 2010).

ModFs may alter the dose-response of the KE in a variety of ways. A selection (not inclusive) of ModFs in humans is provided in Table 1.

Both the KEDRF and Q-KEDRF represent an evolution of the MOA/HRF. Thus, both frameworks assume that sufficient evidence exists to posit the MOA under consideration and to identify hypothesized KEs based on this evidence (Boobis et al. 2006, 2008, 2009, Meek 2008, Meek et al. 2003, Seed et al. 2005, Sonich-Mullin et al. 2001).

If a putative MOA cannot be established, then the Q-KEDRF will not be applicable. Nonetheless, a risk assessment, albeit bearing greater uncertainty, can still be attempted using

other methods such as margin of exposure evaluation based on the most appropriate endpoint (Figures 1 and 2, Box B1).

A sequence of KEs represents a progression over both dose and time. Knowing the relationship between the various KEs in both dose and time along with an understanding of the underlying biology will contribute to the understanding of the role of particular KE within the MOA. Often, counterfactual information is not available. It may be very difficult to demonstrate the necessity of a particular proposed KE. Understanding the biology can help, but conclusive support of necessity will be a data gap.

Identifying a KE is based on the confidence one has that this event is necessary for the apical event/adverse outcome and is based on an overall WoE evaluation of qualitative and quantitative aspects of the MOA as well as whether the hypothesized roles of the KEs are consistent with the biological basis of the adverse outcome.

The Hill considerations have been adapted for use in understanding MOA. Hill (1965) termed these "viewpoints" or "features to consider" rather than true criteria. Hill's considerations are emphatically not a checklist and necessitate rigorous scientific thinking. They have been quite correctly called "guideposts on the road to common sense" (Phillips and Goodman 2006). Hence, the Key Event/Dose-Response Concordance analysis or Dose-time Concordance analysis requires a rigorous and reasoned WoE approach to reach an understanding of the overall MOA (Phillips and Goodman 2004). Very recently, newly evolved rank-ordered Bradford Hill considerations for application in a MOA analysis were developed (Meek et al. 2014a). In rank order, these include biological concordance, essentiality of key events, concordance of empirical observations, consistency and analogy.

For each proposed KE, if removal or blockade of its occurrence could be accomplished (*i.e.*, the counterfactual experiment), then its necessity (or lack thereof) and consequent identity as a KE could be supported. This is the consideration of essentiality. A cause–effect relationship between a chemical and an adverse effect can never be unequivocally proven because causality itself cannot be proven—only inferred with varying degrees of certainty (Adami et al. 2011). A proposed MOA represents a testable hypothesis (Popper 1959) and the KEs as aspects of that testable hypothesis can be examined in a weight of evidence framework to infer causality (Guzelian et al. 2005, Hill 1965, Phillips and Goodman 2004, 2006, Susser 1986).

Therefore, as indicated in earlier publications on MOA, an essential aspect of the process is identification and evaluation of attendant uncertainties. Each step in a MOA analysis should be accompanied by a list of critical and associated data gaps, with a clear indication of those, if filled, likely to have the most impact on the conclusions. The implications of the existing uncertainties should be explored during dose-response assessment.

Relationships between key events, AEs, and the adverse outcome

The development of a proposed or hypothesized MOA will necessitate identification of KEs and understanding of the dose-response and temporal relationships between the various KEs and the adverse outcome as well as between the KEs

Table 1. Modulating Factors (ModFs) potentially affecting KEs for dose-response in humans. ModFs fall into three general categories shown in the left column. The middle column shows subcategories and the right hand column shows some aspects to consider.

Category	Sub-category	Aspects
Host Factors	Genetic Variation	Polymorphisms
	Disease/Illness	Chronic
		Acute
	Defense mechanisms	Immune responsiveness
		DNA repair
		Cell proliferation
		Cell death
	Physiology	Sex
		Life stage
		ADME
		Hormonal status
Life Style	Diet	Calories
		Fat content
	Tobacco	Usage
	Alcohol	Usage
	Exercise	Frequency
		Intensity
	Pharmaceuticals	Usage
	Illegal drugs	Usage
	Dietary supplements	Vitamins
		Anti-oxidants
Environment	Co-Exposures	Duration
		Air
		Water
		Food
		Dust
		Occupational

Table 2. Dose-time concordance table for dimethylarsinic acid.

Time	2 weeks	2–3 weeks	10 weeks	25 weeks	104 weeks
Dose (ppm in diet)	Increasing time				
2	Metabolism*	Metabolism*	Metabolism* Cytotoxicity	Metabolism* Cytotoxicity*	Metabolism* Cytotoxicity*
10	Metabolism*	Metabolism* Cytotoxicity	Metabolism* Cytotoxicity	Metabolism* Cytotoxicity*	Metabolism* Cytotoxicity*
40	Metabolism*	Metabolism* Cytotoxicity	Metabolism* Cytotoxicity Proliferation Hyperplasia	Metabolism* Cytotoxicity* Proliferation* Hyperplasia	Metabolism* Cytotoxicity* Proliferation* Hyperplasia Carcinomas
100	Metabolism*	Metabolism Cytotoxicity Proliferation Hyperplasia	Metabolism Cytotoxicity Proliferation Hyperplasia	Metabolism Cytotoxicity* Proliferation Hyperplasia	Metabolism* Cytotoxicity* Proliferation* Hyperplasia Carcinomas

The asterisk means that the key event has not been observed at the specific dose/time point but is presumed to have occurred. Although not used here, shading of the table may be helpful with a shading scheme based on the number of KEs. Figure 5 in Meek et al. (2014b) provides another organizational scheme for the dose-time concordance table (Please see Figure 3 for the MOA and text for details).

themselves. This is the purpose of the Dose-Time Concordance table (Table 2). Such a table also addresses the temporal aspects of Box B3 in Figure 2 (Meek et al. 2014b).

In 2005, EPA's Office of Pesticide Programs proposed a MOA for the carcinogenesis of dimethylarsinic acid or DMA^V, also known as cacodylic acid (USEPA 2005c). DMA^V administered in the diet or drinking water produced bladder cancer in rats. There are four KEs in the MOA for bladder tumors in rats; these are: (1) generation of the reactive metabolite trivalent DMA (DMA^{III}) that is dependent on DMA^V and can be observed as the urinary excretion of trivalent DMA greater than 0.1 μM in urine; (2) cytotoxicity occurring within the superficial epithelial layer of the urinary bladder; (3) consequent regenerative proliferation; and, (4) hyperplasia of the urothelium (Cohen et al. 2006, USEPA 2005c). The qualitative relationships between these KEs in both dose and time is shown in Table 2, which is an example of the dose–time concordance table (Meek et al. 2014a, Meek et al. 2014b).

In two-year bioassays, dietary administration of 9.4 mg/kg/d DMA^V produced a statistically significant incidence of tumors; dietary administration of 4.0 mg/kg/d produced a statistically significant incidence of hyperplasia. There were no histopathological changes in the urothelium observable using light microscopy from dietary administration of 1 mg/kg/d or lower. In shorter term mechanistic studies using light and scanning electron microscopies to detect superficial cytotoxic changes, evidence of cytotoxicity was present at dietary doses of 1 mg/kg/d and higher. These same mechanistic studies used bromodeoxyuridine (BrdU) labeling index to assess cell proliferation and observed an increase in proliferation at a dietary dose of 1 mg/kg/d and above.
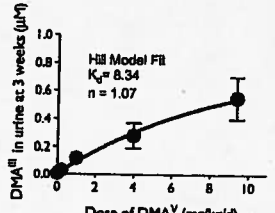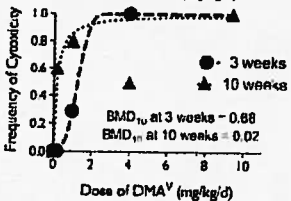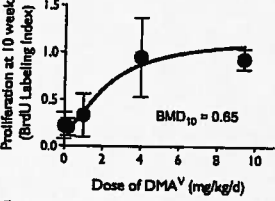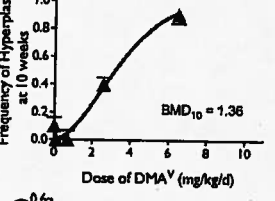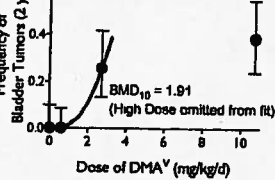
In rats administered DMA^V in drinking water, genomic microarray analysis revealed a change in the pattern of altered gene expression between 0.4 and 4.7 mg/kg/d, the same level at which an apparent threshold was observed using transmission electron microscopy (Sen et al. 2005). Critical cytotoxic urinary levels of the reactive metabolite DMA^{III} were present in rats

orally administered DMA^V at doses of 1 mg/kg/d and above, but absent at 0.2 mg/kg/d. The level of detection for DMA^{III} in urine was 0.01 μM (USEPA, 2005c).

Evidence strongly suggests that DMA^{III} is not DNA reactive, and likely is not genotoxic except at relatively high concentrations (Cohen et al. 2006). Table 2 summarizes the dose-response and temporal relationships for each of the KEs. For risk assessment purposes, it is reasonable to base the assessment on the most sensitive of the KE changes, that is, cytotoxicity. Based on such an analysis, the no-observed-adverse-effect level (NOAEL) is 0.2 mg/kg/d via diet. Similar findings have been identified in rats administered DMA^V in the drinking water (Cohen et al. 2006). Table 3 shows an example of the Dose-Response Species Concordance table that supports quantitative interspecies extrapolation of KEs.

Although the dose-response for humans in Table 3 is lacking, toxicokinetic interspecies extrapolation could be based on differences in the metabolism and kinetics of DMA^V in rats and humans. The evidence indicates that DMA^V is a poor substrate for the methylating enzyme for arsenicals in humans (As^{3+} methyltransferase, As3mt) whereas in rats, this enzyme can readily methylate DMA^V to trimethyl arsenic oxide (Thomas 2007). A physiologically based pharmacokinetic (PBPK) model for DMA^V could support further refinement of the risk assessment, but such a model was not fully developed in 2005 (Evans et al. 2008, USEPA, 2005c). In vitro cytotoxicity assays utilizing rat urothelial cells showed an effect at concentrations of approximately 0.2 μM or higher; in comparison, in vitro human urothelial cells showed less sensitivity, with cytotoxicity produced at concentrations of 0.5 μM and higher (Cohen et al. 2006). Hence, overall, humans would be less susceptible than rats based on both kinetics and dynamics. These quantitative differences could potentially be used to develop a data-derived species extrapolation factor or chemical-specific adjustment factor (USEPA 2011, WHO-IPCS 2005, Meek et al. 2014b).

Table 3. Dose-Response Species Concordance Table for Key Events (KEs) in the MOA of dimethylarsinic acid (DMA^V) (Adapted from USEPA, 2005c).

| Event or factor | Qualitative concordance | | | Quantitative concordance and quantitative Dose-response | | |
	Animals	Humans	Concord-ance	Str.*	Animals	Humans
Key events						
Key Event #1 Metabolism to DMA^{III}	DMA^{III} detected in urine following 26 weeks treatment with 100 ppm DMA^V	Evidence following DMA^V exposure too limited to draw conclusions, but DMA^{III} shown to be present following human exposure to iAs	Plausible	+/−	Hill Model Fit K_d = 8.34 n = 1.07 (DMA^{III} in urine at 3 weeks (μM) vs Dose of DMA^V (mg/kg/d))	NA
Key Event #2 Urothelial Cytotoxicity	Urothelial toxicity observed in vivo in rats at 2 ppm but not enough for successive key events	Potential to occur in humans but unknown if sufficient DMA^{III} formed	Plausible	+/−	Frequency of Cytotoxicity vs Dose of DMA^V (mg/kg/d); 3 weeks, 10 weeks; BMD$_{10}$ at 3 weeks = 0.68, BMD$_{10}$ at 10 weeks = 0.02	NA
Key Event #3 Urothelial Proliferation	observed at 0.5 mg/kg/d DMA^V	Potential to occur in humans but unknown if sufficient DMA^{III} formed	Plausible	+/−	Proliferation at 10 weeks (BrdU Labeling Index) vs Dose of DMA^V (mg/kg/d); BMD$_{10}$ = 0.65	NA
Key Event #4 Hyperplasia	observed at 2 mg/kg/d or 0.3 to 2 μmol DMA^{III} in urine	Potential to occur in humans but unknown if sufficient DMA^{III} formed	Plausible	+/−	Frequency of Hyperplasia at 10 weeks vs Dose of DMA^V (mg/kg/d); BMD$_{10}$ = 1.36	NA
Apical Event Tumors	observed at 5 mg/kg/d DMA^V or 0.8 to 5.05 μmol DMA^{III} in urine	No data in humans	Concordance cannot be made because there is no human data	−	Frequency of Bladder Tumors (2 yr) vs Dose of DMA^V (mg/kg/d); BMD$_{10}$ = 1.91 (High Dose omitted from fit)	NA

*Str. = strength.

In such a case, this information could be added to the Dose-Response Species Concordance Table.

Low protein or vegetarian diets decrease the availability of S-adenosyl methionine (SAM), and arsenic methylation uses SAM as a methyl donor. Hence, diet may constitute a ModF to be considered (Gamble and Hall 2012).

The risk assessment conducted by EPA's Office of Pesticide Programs (OPP) used a benchmark dose lower confidence limit of 0.07 mg/kg/d DMA^V based on cell proliferation as the 1% point of departure (USEPA 2005c) and a nonlinear low-dose extrapolation to develop a reference dose protective of cancer based on this MOA. Here, this example serves to demonstrate the use of the Dose-Time Concordance Table (Table 2) and the Dose-Response Species Concordance Table (Table 3). The BMD information for KEs occurring at 10 weeks—cytotoxicity, proliferation, and hyperplasia—provided a way to order these KEs and supports their order in the dose–time concordance table (Table 2).

An example of how to use the RISK21 exposure-toxicity matrix is provided (Figure 3). The heavy dotted line on the matrix represents a hazard quotient (HQ) of one. The blue square represents the intersection of exposure and toxicity. If any part of this area extends above the line representing an HQ of one, then exposures may be of concern. In the case of cacodylic acid, all exposure levels within the range of chronic dietary exposures are less than the RfD (USEPA 2006). The exposure-toxicity matrix is flexible; in addition to the range shown here, probability distributions of exposure and/or toxicity can be shown as a means of visualizing probabilistic characterizations of exposure, toxicity, and risk.

Concordance of the MOA between humans and animals

The human relevance of a hypothesized MOA may depend on both qualitative and quantitative factors. As evident from the example with DMA^V above, EPA's Office of Pesticide Pro-
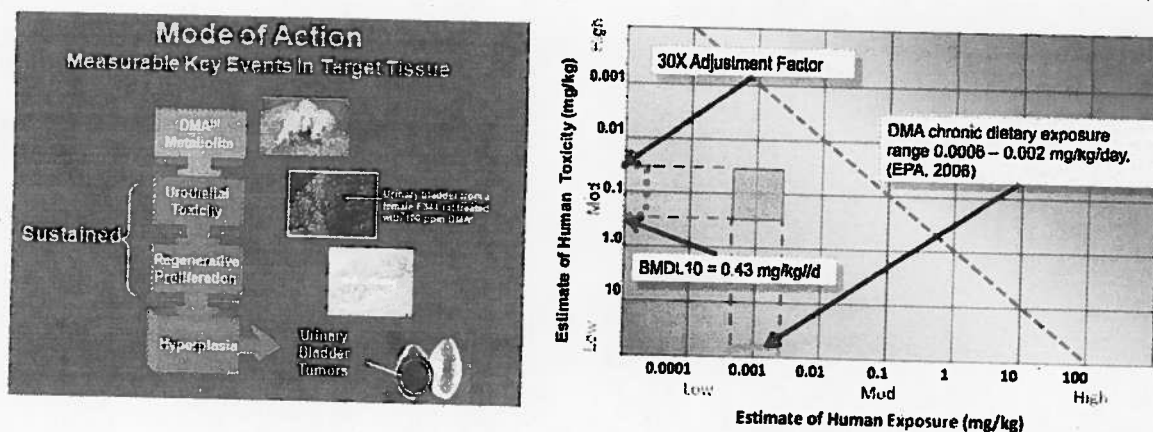
Figure 3. Use of MOA in the HESI RISK21 Matrix. Left: MOA for Tumor Induction by Dimethylarsinic Acid (DMA^V; Cacodylic Acid) that includes cytotoxicity, regenerative proliferation, and hyperplasia. This MOA is used to illustrate the dose-time concordance table and dose-response species concordance table (Tables 2 and 3). Right: Matrix showing the exposure estimates and toxicity range (BMDL$_{10}$ to RfD) for chronic dietary exposure, data from EPA, 2006.

grams clearly recognizes this fact and the need for assessing both qualitative and quantitative concordance of KEs between animals and humans (Dellarco and Fenner-Crisp 2012). For example, in the early 1990s, a technical panel from EPA concluded that male rat renal tubule tumors from chemicals that induced accumulation of α_{2u} globulin were likely not relevant to humans based on qualitative considerations (Rodgers and Baetcke 1993). Naphthalene produces respiratory tract tumors in rats, but the MOA for these tumors in rats is based on metabolic enzyme activity that is not present in humans (Piccirillo et al. 2012).

The Dose-Response Species Concordance Table (Table 3) is a means of illustrating the similarities and differences in a proposed MOA between humans and the test-species. Likely other information, narrative and/or additional tables, will be needed to provide all the information needed for species extrapolation.

Qualitative concordance of key events between humans and animals

Human relevance of the apical endpoint is best determined using a hypothetico-deductive WoE approach (Boobis et al. 2006, 2008, Meek et al. 2003, Rhomberg et al. 2010, Seed et al. 2005, Sonich-Mullin et al. 2001). To address human relevance of the MOA, qualitative concordance between humans and animals for each KE needs to be considered. *In vitro* data from human or animal cells or tissues and/or *in silico* data may also be available; these data play a useful role in the determination of concordance as well. Ideally, the data will be sufficient to determine which of the KEs is relevant to humans, and these data may thus be used to support statements about the relevance to humans of the hypothesized MOA in animals.

Quantitative concordance of the MOA between humans and animals

Quantitative examination of both the dose-response and timing of KEs is also necessary to determine human relevance. For example, a MOA may be operative in both animals and humans, but extremely unlikely in humans because of quantita-

tive toxicokinetic or toxicodynamic differences. If the KE has the potential to occur in humans, then this quantitative examination can be used to inform animal-to-human extrapolation. Hence, the quantitative concordance should provide information about the EC$_{50}$ and/or point-of-departure values for as many KEs as possible in both humans and the animal test species. Including NOAELs or other measures of the no-effect level/threshold such as that defined using the EC$_{05}$ baseline projection method of Silkworth et al. (2005) or the "hockeystick" fitting method of Lutz and Lutz (2009) may also be useful.

The role of quantitative dose-response information

For dose-response assessment, it can be extremely useful to examine quantitative dose-response information from as many relevant sources as possible (*e.g.*, human, laboratory animal or *in vitro* data). These data will help inform the progression of events within the MOA. *In vitro* to *in vivo* extrapolation (IVIVE) may be necessary to express the dose-response for *in vitro* data on a similar dose scale as the *in vivo* data. Where possible, the actual dose-response plots should be shown. It is often helpful to show the dose-response of a KE and that of the apical event or adverse outcome on the same plot (*e.g.*, Figure 2 in Simon, et al 2009). Once the MOA for rat liver tumor promotion by TCDD was considered, the task of arranging the dose-response plots in a figure that displayed the MOA in a meaningful way became easy. Rodent liver tumor promotion is one of the longest and most intensively investigated MOAs in toxicology (Budinsky et al. 2014). Developing similarly informative figures may not be as easy for less well-studied chemicals. Figure 8 is an attempt to create a similar figure for the uterotrophic response. For clarity, it is helpful to have the same dose range on the x-axis in all the plots. When not possible to provide plots of dose-response curves, sufficient narrative should be presented to explain animal/human similarities and differences. If sufficient data in both dose and time are available for a particular KE, a three-dimensional graph with an interpolated surface plot that shows the occurrence of the KE along both dose- and time-axes may be very informative (Box 2; Budinsky et al. 2014).

Use of dose-time and dose-response concordance information in understanding the MOA

In general, events that occur at low doses and/or at early stages in the progression toward the apical event may represent:

- the start of a temporal progression;
- the initial stages of a developing change; or,
- a factor that potentially causes other KEs that occur at higher doses or at a later time in the progression.

Generally, demonstrating that a particular event is necessary is experimentally difficult; yet, it may be possible in some cases (*e.g.*, with transgenic or knockout animals), thus providing a powerful counterfactual demonstration supporting the identification of the event as a KE (Phillips and Goodman 2006). In the example used in Table 2 and Figure 3, let us assume that blocking metabolism of DMA^V or cacodylic acid to dimethyl arsinous acid (DMA^{III}) could reduce or alleviate the KE of urothelial cytotoxicity. The enzyme arsenic methyltranferase (As3mt) catalyzes all steps in the metabolic pathway from arsenite to mono, di, and trimethylated arsenic compounds (USEPA 2005c). If cytotoxicity and tumors did not occur when As3mt was inactivated, this would confirm the role of metabolism and resulting cytotoxicity as necessary and thus as KEs; conversely, if cytotoxicity and tumors occurred even when As3mt was inactivated, one could no longer support the identification of metabolism and cytotoxicity as KEs. Once the DMA^{III} is formed, it readily reacts with free sulfhydryl groups. Co-administration with high doses of a sulfhydryl-containing chemical, such as dimercaptopropanesulfonic acid (DMPS) can act as a trap for the DMA^{III}, reduce or prevent its reaction with proteins, and thus reduce or prevent its biological effects. Co-administration of DMA^V with DMPS inhibits the induction of cytotoxicity and regenerative proliferation of the urinary bladder, providing evidence for DMA ^{III} as the reactive intermediate and AE/KE in the DMA^V-induced bladder cancer in rats (Cohen et al. 2006).

The exact nature of a KE cannot be necessarily understood from either its dose-response or its timing of occurrence. For example, some early KEs may need to be sustained in order for later KEs or the apical event/adverse outcome to occur (*e.g.*, Budinsky et al. 2014).

Toxicokinetics may affect this timing. For example, lipid soluble chemicals may be stored in adipose tissue for months or years and produce effects on an ongoing basis; for similar reasons, the dose of a bioaccumulative chemical may be measured as body burden or tissue concentration. In such a case, the area under the curve (AUC) in units of concentration × time would likely represent the ongoing accumulation in both dose and time better than body burden or tissue concentration at a single time point. Sequestration of a chemical by protein binding may also be represented best by the AUC. A monotonic dose-response relationship between the AUC and a biomarker for a putative KE such as enzyme induction indicates that exploring the quantitative relationship between this biomarker and the apical event/adverse outcome may likely help elucidate details of the MOA.

In other cases, the occurrence of some early KEs may trigger a cascade of other events. These early KEs either resolve themselves or are no longer empirically observable. However, the cascade of triggered events continues and leads ultimately to the adverse outcome/apical event. An example of such an effect is illustrated by the difference between long-acting and short-acting estrogens; short-acting estrogens produce early but not late events in the uterotrophic response whereas long-acting estrogens produce both. Estradiol, a long-acting estrogen, can stimulate uterine growth for up to 72 hours whereas the effects of estriol, a short-acting estrogen, last only 24 hours. In fact, estriol and other short-acting estrogens may display partial antagonism when continuously administered in longer-term assays (Clark and Markaverich 1984). Again, these various estrogenic compounds show differences in their dose-response over time.

The Q-KEDRF toolbox

Quantitative methods are often a good way to understand modulating factors. When a sufficient number of experiments determine the procession/cascade of KEs on both dose- and time-scales, quantitative methods are less necessary to obtain an understanding of the MOA. In such cases, the Dose-Time Concordance Table will suffice, and such was the case for DMA.

The relationship of KEs to the critical effect/apical or adverse outcome can be understood by expressing the tumor BMD as a multiple of the BMD values of various KEs (*e.g.*, Simon et al. 2009). BMD_{10} values are shown on the figures in Table 3. Values for the BMD multiple for the three KEs, cytotoxicity, proliferation and hyperplasia, can be determined as:

$$\text{BMD Multiple} = \frac{\text{BMD}_{\text{ApicalEvent}}}{\text{BMD}_{\text{KeyEvent}}} \quad (1)$$

Using Eq. (1), one can determine that the tumor POD is almost 100 fold greater than the BMD_{10} for cytotoxicity at 10 weeks, about 3 fold greater than the BMD_{10} for proliferation at 10 weeks, and about 1.5 fold greater than the BMD_{10} for hyperplasia at 10 weeks. These values provide a means of judging the relative position of the various KEs along the dose continuum.

Quantitative dose-response methods also may prove very useful for understanding and refining proposed MOAs. For example, Simon et al. (2009) used both potency and steepness to determine the dose progression of likely KEs in the MOA for rodent liver tumorigenesis by dioxin. This approach was used again to examine nuclear receptor activation leading to tumor promotion (Budinsky et al. 2014, Corton et al. 2014).

While no single method is appropriate for all situations, the methods described in this section are all part of the Q-KEDRF toolbox. Contrast tests and regression analysis using well-established statistical methods may prove useful for ordering events within a hypothesized MOA (Bretz et al. 2005, Sawilowsky 2002, Tukey et al. 1985). Lutz and Lutz (2009) provide full details of their "hockey stick" model and an R script for ease of use. For developing dose levels corresponding to specified response levels (i.e., benchmark doses), Murrell et al. (1998) suggest the use of the calculated slope of the dose-response and baseline projection. Silkworth et al. (2005) implemented a form of this method but did not describe details of their calculation. The method was fully developed, including

calculation of confidence intervals in Budinsky et al. (2010). Sand et al. (2006) used the second and third derivatives of the dose-response function to obtain a "transition dose range." Further, they identified a response level of 21% as the transition point for the Hill model.

Naïve practitioners may be tempted to use of the numerical results of a single method as a quantitative threshold. In this regard, any quantitative estimate of a threshold needs to be considered in the light of biological significance, and quantitative estimates of thresholds and transitional dose values (TDVs; see Section 4 below) from a variety of methods should be developed (Budinsky et al. 2010). The discussion of thresholds in Slob and Setzer (2014) is particularly enlighting. Notable is their argument that dose is better represented on a logarithmic scale than on a linear one. The use of logarithms with dose is consistent with thermodynamic principles (Waddell 2005, Waddell 2008). This caveat notwithstanding, the ability to obtain quantitative dose values within the low-dose region can greatly help determine the order in dose and time of events within a hypothesized MOA (See Supplementary Content for an example).

Modulating factors—accounting for variation within the human population

The application of the MOA/HRF and the QKEDRF can provide informative and quantitative descriptions of the MOA and dose-response for adverse outcomes (cancer and non-cancer) including those at low, environmentally relevant exposure levels. Such an approach is essentially designed to describe the form of the dose-response curve for a generalized population. What is also needed is an approach that allows for incorporation of the influence of ModFs on the dose-response of KEs that will ultimately enable the quantitative population-level assessment of risk at low exposure levels. ModFs should be understood in terms of their effects on biological processes and KEs within an MOA. The effect of a low protein vegetarian diet on the availability of S-adenosyl methionine as a possible ModF for the toxicity of DMA^V has already been discussed.

One universal ModF is likely to be individual variation in reserve capacities, for example, differing amounts of reduced glutathione that affect the occurrence of particular KEs between individuals and over time within a single individual. Other examples would be the expression of the p53 gene product or the occurrence of oxidative DNA damage.

Variations in the intracellular level of a large number of transcription factors and cofactors can alter both the efficacy and potency for both steroid and glucocorticoid hormones (Blackford et al. 2012, Simons 2010, Sun et al. 2008, Zhang et al. 2012). In fact, limitations in the amount of coregulatory proteins available within the transcription complex may lead to non-monotonic dose response curves such as squelching (Charlier 2009, Kraus et al. 1995, Zhang and Teng 2001). Graphical analysis of these changes yields valuable mechanistic information when the production of the apical response follows a first-order Hill dose-response curve (Dougherty et al. 2012, Ong et al. 2010, Simons and Chow 2012). However, regardless of the order of the dose-response curve of the adverse outcome/apical response, the magnitude and/or position of the dose-response curve will likely be similarly

modified by any chemical that binds to nuclear receptors and/or other transcription cofactors.

There may exist many potential ModFs for any particular exposure scenario (*e.g.*, specific chemical, type of exposed individual or group). Therefore, organizing these factors based on common biological mechanisms would be helpful. By doing so, the likelihood of a ModF affecting a particular MOA could be determined. One approach described here is to identify a list of general ModFs that can be broadly separated as Host, Life Style and Environment (Table 1). Other classification schemes for ModFs, perhaps based on MOA, will likely emerge as risk assessment practitioners gain experience with the Q-KEDRF. The OECD is currently developing a program on AOPs, and the International QSAR foundation is developing an "Effectopedia" to provide information about AOPs/MOAs as part of a global scientific collaboration; the Q-KEDRF will likely interface quite well with these efforts (Ankley et al. 2010, Patlewicz et al. 2013). The use of the term "Initial Molecular Event" (IME) to refer to the first step Event, as suggested by Patlewicz et al. (2013), is appropriate and conveys an accurate message—that the initial event may not obligatorily lead to the adverse outcome.

Examples of modulating factors

Two examples are presented below with the goal of improving the understanding of how ModFs can affect KEs and potentially impact the dose-response for the adverse outcome. These examples illustrate different aspects of KEs within biological pathways: xenobiotic processing (metabolism) and endocrine stimulation.

Example 1: Genetic variation in PON1 potentially modulates chlorpyrifos metabolism and toxicity

The MOA for OPs is well known—inhibition of cholinesterases with toxicity manifested as central and peripheral cholinergic effects (Figure 4) (Mileson et al. 1998). Cholinesterase inhibitors include carbamate insecticides, physostigmine used to treat glaucoma, and Δ^9-tetrahydrocannabinol, the active moiety in marijuana. Paraoxonase 1 (PON1) is an arylesterase that metabolizes organophosphate compounds (OPs). Thionophosphorus OPs such as chlorpyrifos (CPF) are metabolized to the oxygen analog or oxon by CYP450 mixed function oxidases. These oxons are potent inhibitors of acetyl cholinesterase (AChE). CPF oxon is inactivated by PON1 in the liver and other tissues (Smith et al. 2011, Timchalk et al. 2002a; 2002b).

Host factors—genetic variability and lifestyle factors. In humans, PON1 activity is age-specific, increasing about 3.5 fold between birth and 7 years of age, remaining constant thereafter (Figure 5) (Smith et al. 2011). Genetic polymorphisms exist in the coding regions of PON1 gene with consequent variation in catalytic activity. For example, PON1 polymorphism at amino acid 192 [glycine (Gln; Q allele) to arginine (Arg; R allele) substitution] changes PON1-mediated esterase activity depending on the substrate present (Adkins et al. 1993). PON1 (R192) hydrolyzes CPF oxon more efficiently than PON1 (Q192) (Richter et al. 2009). Along with the general increase in activity with age, differing phenotypes mature at different rates (Huen et al. 2010). Polymorphisms exist in the
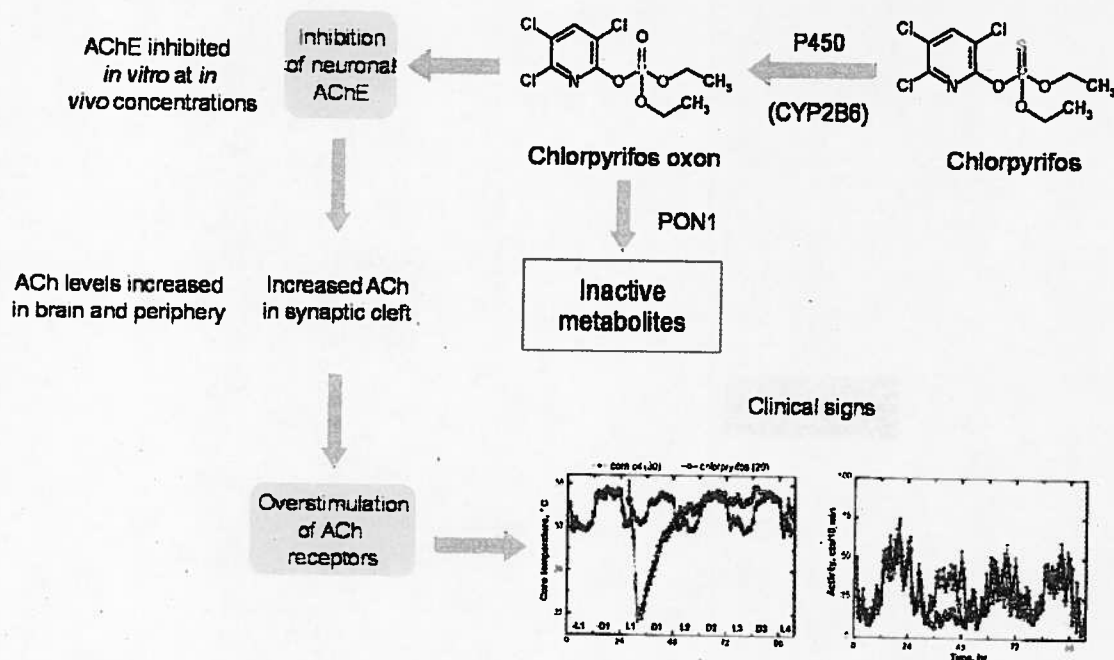
Figure 4. Mode of Action of Chlorpyrifos showing metabolic activation to CPF-oxon and inhibition of acetylcholinesterase as the critical effect. (Figure courtesy of Dr. Alan Boobis).

promoter region of PON1 and may affect expression level and tissue activity. A single nucleotide polymorphism located 108 bases before the transcription start site ($PON1_{-108}$) accounts for 22.4% in the variability in arylesterase activity (Brophy et al. 2001, Deakin and James 2004). Overall, an individual's

Figure 5. PON1-mediated Vmax values vs. age (upper plot). PON1 functional phenotypes are represented by open circles, open triangles, and open squares for QQ, QR, and RR, respectively (see text for definitions). CPF- oxon hydrolysis V_{max} values in plasma over paraoxon hydrolysis activity (lower plot) resolves QQ and QR, but not QR and RR. (From Smith et al. 2011; permission to reproduce figures granted by Dr. Jordan Smith, 22 March 2013.).

PON1 activity is dependent on variations in the coding region as well as the promoter region. Both the polymorphisms and the age-dependent increase in activity would be categorized as host factors. The age-dependent increase in V_{max} in plasma PON1 activity on a plasma volume basis for individuals of all three genotypes (QQ, QR and RR) is shown in Figure 5.

In addition to these host factors, a number of lifestyle factors affect PON1 activity. Statins are cholesterol-lowering substances that occur naturally in red rice yeast and are also prescribed as drugs. In some human studies, very modest increases in serum PON1 have been observed in those taking statins. However, in other studies, no effect is seen (Costa et al. 2011). Moderate alcohol consumption appears to increase serum PON1 (Sierksma et al. 2002). Pomegranate juice contains several polyphenols and its consumption increases plasma PON1 activity in normal humans and in diabetic patients (Aviram et al. 2000, Rock et al. 2008). The lifestyle factors increase PON1 activity and would tend to desensitize individuals to the effects of thionophosphorus OPs.

Consideration of modulating factors in a chlorpyrifos risk assessment. For risk assessment purposes, the question that must be asked is whether changes in PON1 actually translate into changes in sensitivity, and, if so, whether these host and/or lifestyle factors produce sufficient variation in PON1 activity such that individuals with a sensitive phenotype such as QQ or the very young might constitute an at-risk subpopulation.

When workers exposed to CPF during manufacture were compared to a referent group of chemical workers, no effect of PON1 phenotype was observed (Albers et al. 2010, Garabrant et al. 2009). Urinary 3,5,6-trichloro-2-pyridinol (TCPy) is a metabolite of CPF and a specific biomarker of exposure (Alexander et al. 2006); TCPy levels in all exposed workers were less than those paralleling previously determined no-observed-effect levels for red blood cell (RBC) AChE inhibi-
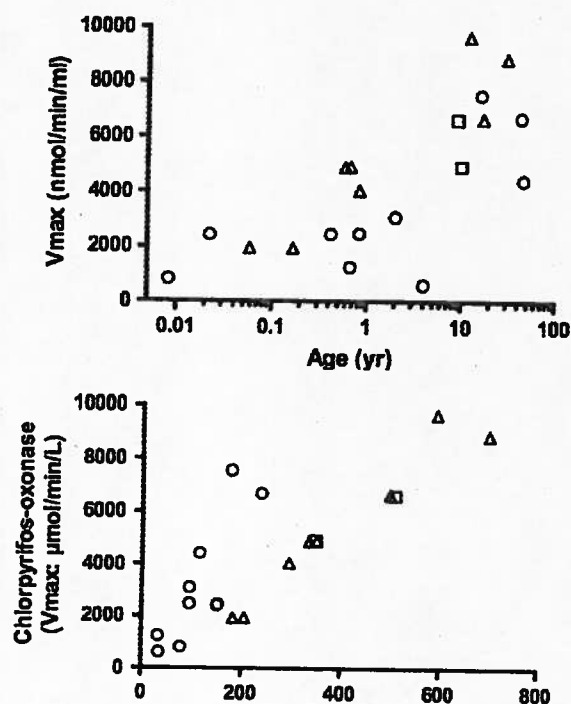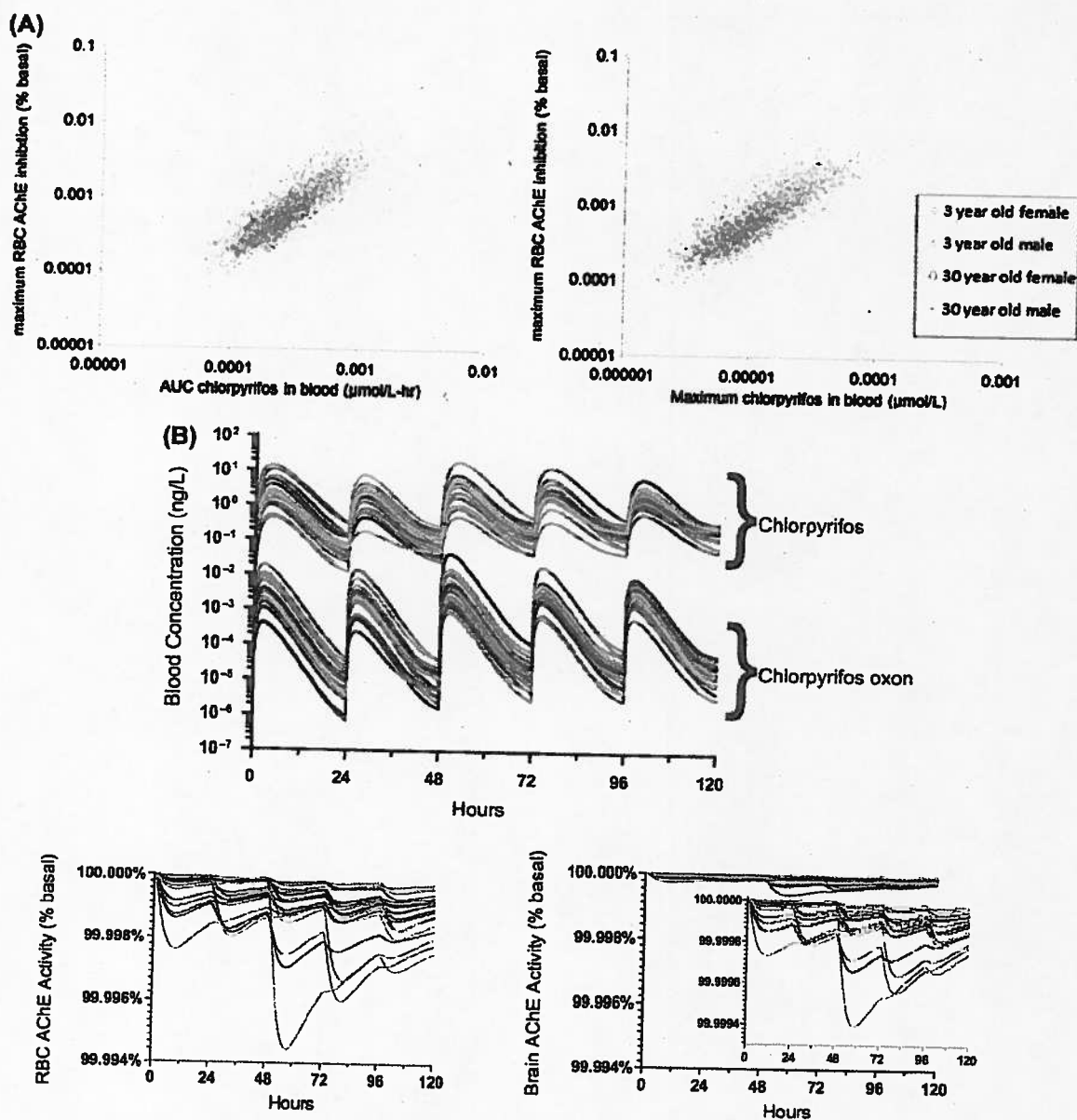
Figure 6. Modeled chlorpyrifos pharmacokinetics in adults and children and resulting AChE inhibition in erythrocytes. A. RBC AChE inhibition from the AUC (left) and maximum CPF concentrations (right) in blood (from Hinderliter et al. 2011). B. Modeled time courses of CPF and CPF oxon in blood from dietary exposures (upper panel) and corresponding RBC AChE inhibition (lower panel). (Reprinted from *Regulatory Toxicology and Pharmacology* (Hinderliter, P.M., Price P.S., Bartels M.J., Timchalk C., Poet T.S. 2011. Development of a source-to-outcome model for dietary exposures to insecticide residues: An example using chlorpyrifos, Regul. Toxicol. Pharmacol. 61, 82–92) with permission from Elsevier.).

tion and changes in neurological function (Albers et al. 2004a; 2004b; 2004c; 2007, 2010, van Gemert et al. 2001).

Enzyme kinetics of PON1 were analyzed in liver microsomes and plasma in both children and adults to measure quantitative age-dependent differences (Smith et al. 2011). These data were incorporated into a probabilistic physiologically based pharmacokinetic and pharmacodynamic (PBPK/PD) model for CPF (Price et al. 2011, Timchalk et al. 2002a, Timchalk et al. 2002b). With this model, the relationship between urinary TCPy and either plasma butyrylcholinesterase (BuChE) or RBC AChE was determined and related to the exposure to CPF. Model results are shown in Figure 6. In three-year-old children, the greatest percent reduction in ChE levels for typical dietary intake was 0.001%. In addition, a sensitivity analysis of the PON1 parameter in blood and liver revealed

only a modest influence of this factor. The presence of other detoxification enzymes established a lower limit for the effect of PON1 variation (Hinderliter et al. 2011, Price et al. 2011).

In contrast, at a dose of 300,000 ng/kg/d of CPF, typical of a high-dose animal study, the model indicated that both the age-dependence and the polymorphisms in the activity of hepatic PON1 would be reflected by substantial differences in RBC AChE levels; however, neither these age-dependent differences nor PON1 enzyme polymorphisms are likely to affect RBC AChE levels at real-world human exposure levels (Garabrant et al. 2009, Hinderliter et al. 2011, Smith et al. 2011, Timchalk et al. 2002a; 2002b).

To incorporate ModFs into risk assessment, the effect of these factors needs to be considered at the point of departure or at current exposure levels and not in a purely

abstract way. An effect of human variation in PON1 on RBC AChE inhibition was observed in the model output at a dose of 300,000 ng/kg/d of CPF but not at current dietary exposures of children and adults for which the respective doses are estimated to be less than 11 ng/kg/d and 3.4 ng/kg/d. Increased sensitivity was not observed at dietary exposures because the exposures were too low to produce a biologically meaningful change in the activity of various cholinesterases, even in sensitive individuals. In addition, individuals of the RR phenotypes appear to have higher activity of PON1 in plasma, thus providing similar capacity for clearance (Figure 5; Smith et al. 2011). Therefore, while the presence of polymorphisms and the age-dependence of PON1 provide illustrations of potential ModFs, the actual effects of these factors must be considered in the context of the entire dose-response curve and relevant exposure levels.

This examination of the MOA for CPF-inhibition of AChE includes tiers 1 through 4 of toxicity resources in the RISK21 roadmap (Figure 1). *In vitro* and *in vivo* data from humans were included; a PBPK/PD model was used for IVIVE and the Q-KEDRF was used to evaluate the ModFs of age and genetic polymorphisms. This probabilistic model is an excellent example of the use of quantitative MOA information in a risk assessment.

Table 4 provides an example of the Species Concordance table for ModFs and presents some of the information discussed above. The table format is sufficiently flexible to accommodate both qualitative and quantitative information. Although the information for CPF was obtained from humans, the columns for animals represent placeholders for those situations in which species extrapolation of the effect of ModFs needs consideration.

Example 2: Factors that can modulate the uterotrophic response

Estrogens induce uterotrophy through activation of the estrogen receptor alpha (ERα), a ligand-activated nuclear receptor and transcription factor. Cellular and physiological factors can modulate the estrogen dose-response for ERα activation, subsequent KEs, and uterine weight gain, the latter considered to be the critical effect in this example. A positive uterotrophic response for a chemical indicates a potential for endocrine disruption (OECD 2003).

Progesterone opposes estrogenic effects and reverses estrogen-induced uterotrophy. Progesterone stops cell growth

and prevents the uterine lining from shedding. Like estrogen, progesterone is a ligand that activates a transcription factor. All transcription factors require cofactors for transcription to occur. One function of these cofactors is to increase the activity of RNA polymerase II, sometimes by facilitating chromatin remodeling and RNA polymerase II access to transcriptional start sites. For constitutively expressed genes, chromatin remodeling plays a smaller role than other gene regulatory factors (John et al. 2011). In contrast, RNA polymerase II is already bound at the transcription start site of a large number of other genes and the binding of a transcription factor is the signal for the polymerase to "start" (Levine 2011). Cofactors that interact with both the estrogen and progesterone receptors include steroid receptor coactivator-1 (SRC-1), receptor interacting protein 140 (RIP140), and the histone acetyl transferase chromatin-binding protein/p300 (Kobayashi et al. 2010, Simons 2008, Simons 2010).

Among the mechanisms by which progesterone is proposed to antagonize estrogen actions is by binding to progesterone receptors (PRs) to form complexes that compete with ERαs for cofactors that help mediate and thus increase ERα-mediated gene transactivation (Giangrande et al. 2000, Kraus et al. 1995, Parisi et al. 2009, Wen et al. 1994). In general, the effects of progesterone oppose the effects of estrogen. Thus, the dose-response curve shifts to the right and the system or individual becomes less sensitive to the effects of estrogens. Given that estrogens induce synthesis of PRs, these combined effects may serve as a means of feedback inhibition of estrogen-activated responses.

Uterotrophy as a model system for understanding MOA. Estrogen-induced uterotrophy in rats is an extensively studied response that has been documented to proceed through estrogen binding to the intracellular ERα as the MIE and is a KE in the MOA for the uterotrophic response. The induction of several genes (i.e., ornithine decarboxylase, glucose-6-phosphate dehydrogenase, lactoferrin, c-fos, and uterine peroxidase) occurs in response to estrogen, and these gene expression changes have been proposed as KEs in the MOA of estrogen-induced uterine growth (Figure 7; OECD 2003). Microarray assays have identified various other genes that may also be part of the overall MOA (Heneweer et al. 2007, Naciff et al. 2003).

The effects produced by ModFs shown in Table 5 can modify gene function not only through direct effects on DNA and chromatin but also by altering the strength of the various

Table 4. Dose-response concordance table for Modulating Factors (MFs) in the MOA of chlorpyrifos.

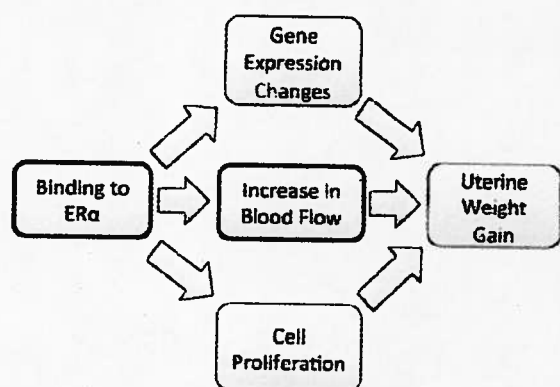| Event or factor	Qualitative concordance			Quantitative concordance and quantitative Dose-response		
	Animals	Humans	Concordance	Strength	Animals	Humans
Modulating Factors MF and affected KE	Animals	Humans	Concordance	Strength	Effects in Animals	Effects in Humans
MF #1 Genetic Polymorphism	NA	R vs. Q allele	NA			QQ genotype more sensitive, but at current exposure levels this difference is not a factor
MF #2 Use of Statin drugs	NA	Statins increase PON1 activity	NA			Statins modestly increase PON1 activity, but the effect is not consistently observed
MF #3 Alcohol Use	NA	Alcohol use increases PON1 activity	NA			This effect is likely not a factor at current exposure levels

Figure 7. Putative MOA for the uterotrophic response.

binding reactions occurring during gene transcription, including interactions between DNA and protein, between RNA and protein, between DNA and RNA, and between various proteins. The effect of these associations on dose-response is not clear at this time. However, the Q-KEDRF approach allows one to test the prediction that chemicals and factors with similar molecular targets will evoke comparable changes in the adverse outcome/apical event.

The rat uterotrophic response to estrogens was selected for a case study of the utility of using a MOA approach. The first step, of course, was to identify KEs or AEs that could serve as biomarkers for these KEs. Given the abundance of experimental data over the years for rat uterotrophy, this task was expected to be a relatively straightforward application of the new framework (Figure 2). OECD (2003) identifies binding to ERα as the MIE and provides a list of early and late events associated with uterotrophy. Unfortunately, dose-response and timing of these early and late events have not been obtained from the same species or preparation and thus, it is difficult to array these in a meaningful Dose-Time Concordance table. However, guidance from OECD as well as the scientific literature was used as the basis of a putative MOA and a set of proposed KEs for uterotrophy (Figure 7). Given the extent of investment in testing for endocrine effects and the relative maturity of the uterotrophic assay, the lack of information from the same or at least comparable studies seems surprising. This situation emphasizes the need to design studies that address the particular question at hand as it relates to elucidation of the MOA, and illustrates how effective the MOA framework can be in rapidly and effectively identifying critical data gaps. Consideration of MOA as early as possible in the risk assessment process would foster the collection of appropriate data to

inform the MOA based on the expected value of the information (Meek et al. 2014a, Meek et al. 2014b). Such an approach would be entirely consistent with the method of problem formulation described in NRC (2009).

Following absorption of estrogen or an estrogenic chemical, binding to ERα would be the MIE. This binding has been measured in a number of species *in vivo* and in cell-free preparations (Levin et al. 1993, Notides et al. 1981). Following receptor binding, early events would include (1) altered expression of estrogen sensitive genes; (2) an increase in uterine blood flow; and (3) an increase in cell proliferation. Respectively, these events can be measured by: (1) microarrays or qRT-PCR; (2) flow transduction or weight gain; and (3) mitotic index or BrdU labeling. Because of the lack of sufficient data from a single high-quality study, as already stated, it is difficult to determine the exact role of these putative KEs in the MOA, but assessing the whole body of evidence using a WoE analysis, KEs can be substantiated. The apical event is, of course, uterine weight gain. At the present time, the order and timing of the changes shown in the third and second columns of Figures 7 and 8, respectively, are not known (Ashby et al. 1999, Gorski et al. 1977, Heneweer et al. 2007, Kaye et al. 1971, Naciff et al. 2003, OECD 2003).

At this point, conclusive identification of putative KEs becomes difficult due to: (1) variations in experimental systems; (2) the absence of data representing multiple KEs from the same study or same laboratory; and (3) and insufficient data points to make quantitative conclusions about dose-response.

Identification of key events for uterotrophy using WoE. Absorption is considered part of absorption, distribution, metabolism, and excretion, and is thus not identified as a KE, although it is the initial event in the process. For some chemicals, metabolic transformation that occurs close in time to absorption may either bioactivate these chemicals to toxic/active metabolites (e.g., polycyclic aromatic hydrocarbons/tamoxifen and cortisone, respectively) or detoxify/inactivate them (e.g., CPF oxon/cortisol) (Chapman et al. 2013, Furr and Jordan 1984). Estrogenic compounds contain one or more phenol groups and, following oral exposure, may be inactivated before reaching the systemic circulation by first-pass phase II metabolism in enterocytes or the liver (e.g., Hengstler et al. 2011). Hence, for estrogenic compounds and uterotrophy, metabolic transformation would not be a KE; however, metabolism may be a KE for other substances that are transformed to toxic metabolites (e.g., dimethylarsinic acid).

For uterotrophy, the MIE of binding to ERα will be a KE if it is empirically observable, and it is very probable that cell proliferation is also a KE. Two KEs can actually be conclusively identified on the basis of counterfactual reasoning and are shown with a thicker outline of the event boxes in Figure 7. The basis for identifying binding to ERα as a KE is the fact that estrogen-receptor knockout mice do not show evidence of cell proliferation, that is, DNA synthesis, in response to estrogen (Curtis et al. 1996, Klotz et al. 2002). However, other responses associated with estrogen-induced uterotrophy such as water imbibition and lactoferrin induction are maintained in the absence of ERα (Das et al. 1997, Winuthayanon et al. 2010). The basis for identifying the increase in blood flow as a KE is the disruption

Table 5. Cellular effects of modulating factors.

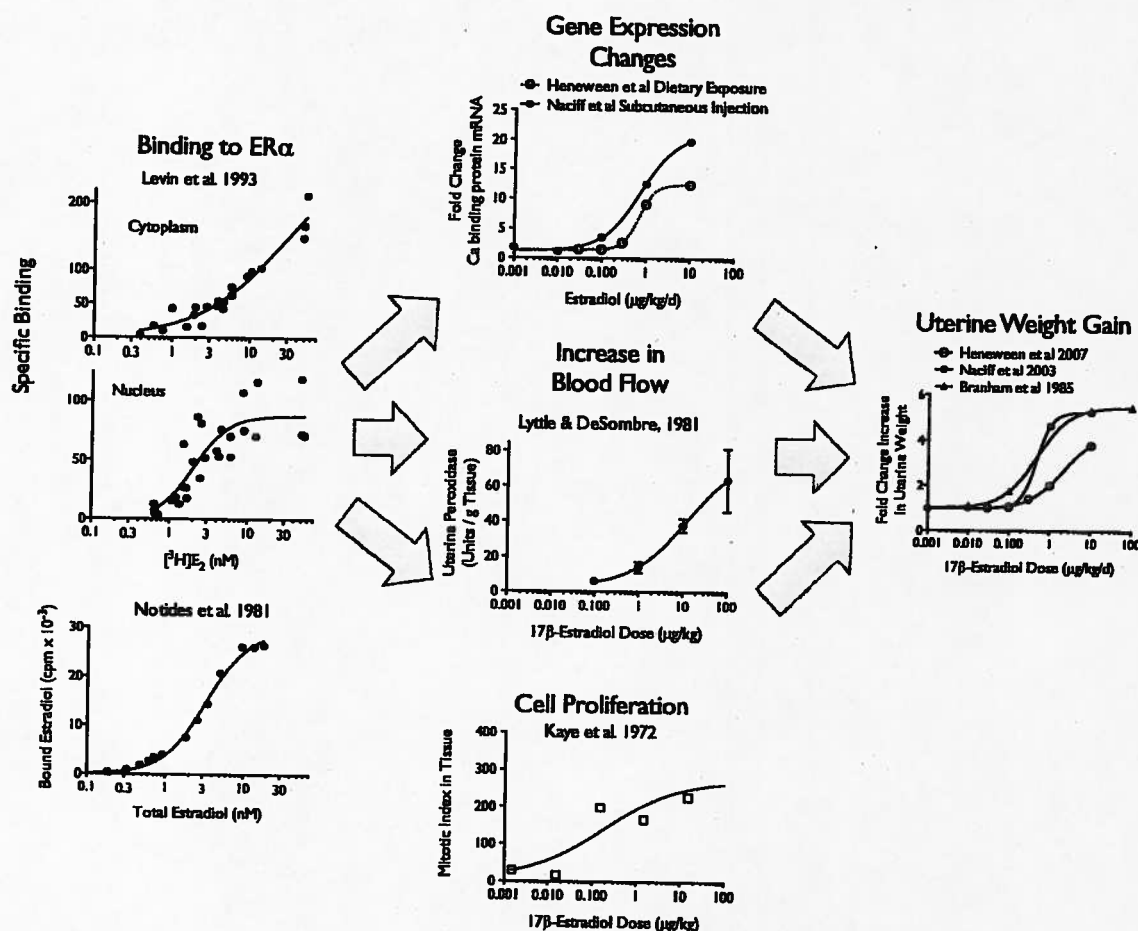| Effect	Sub-effect
Gene Structure	Mutations
	Deletions
	Duplications
Gene Expression	Transcription factors
	Co-activators/accelerators
	Co-repressors/decelerators
	Co-modulators
Post-translational modifications	Acetylation
	Methylation
	Phosphorylation
	Others

Figure 8. Dose-response plots for putative key events in the MOA for the uterotrophic response.

of the uterotrophic response by L-NG-nitroarginine methyl ester (LNAME) that blocks nitric oxide synthase (Rao et al. 1995, Rosenfeld et al. 1996). Alternatively, the production of catechol estrogens due to an estrogen-mediated increase in peroxidase may also contribute to alpha-adrenergic activation, vasodilation of the uterine arteries, and a consequent increase in blood flow (Lyttle and DeSombre 1977, Farley et al. 1992, Stice et al. 1987a; 1987b). In this example, the increase in uterine peroxidase is being identified as an AE to represent the increase in blood flow (Figure 8).

Dose-response modeling can elucidate the MOA for uterotrophy. Table 6 shows values for Hill model fits for the various responses of KEs and putative KEs. When data are available from a single study, both the EC_{50} and the slope of the dose-response curve are important in understanding the MOA and the relationship to the apical response (e.g., Simon et al. 2009).

EPA's Cancer Guidelines (USEPA 2005a) suggest the possibility of using an earlier KE as a precursor to the apical event and developing a toxicity criterion using the dose-response of this KE. Caution is warranted when using a KE as the basis for development of a toxicity criterion when the dose-response of the KE has a higher value of the Hill coefficient than the apical response; steeper dose-response curves (higher Hill coefficients) will have greater nonlinearity than a first-order Hill response and thus, the rising phase of the dose-response may commence at a higher dose value. Therefore, using the

dose-response of the KE as the basis of a toxicity factor may not be a health-protective choice in the case of an apical event or critical effect known to follow a first-order Hill function, as is the case for uterotrophy (OECD 2003). By the same reasoning, the use of an early KE as the basis of a toxicity factor may be inappropriately over-conservative when the KE exhibits a shallower dose-response curve (lower Hill coefficient) than does the critical effect/adverse outcome.

The variation in the Hill coefficients observed in Table 6 is likely a reflection of the fact that these data were obtained from disparate sources. The plots of estrogen binding in the left column of Figure 8 were obtained *in vitro* and thus, IVIVE would be needed to set these on a similar dose scale as whole animal effects.

At this time, most available dose-response curves for estrogen-induced genes and other responses associated with uterotrophy have so few data points that the determination of quantitative aspects of dose-response becomes problematic. Even after all the years of studying uterotrophy, the shape of the curve for the critical effect of uterine weight gain has not been firmly established (Note the variation between the three curves in the rightmost plot of Figure 8).

For these reasons, even the relatively superficial MOA for uterotrophy cannot yet be constructed without new, more detailed data. First, high-quality dose-response curves with more data points for intermediate responses are critical so that an accurate determination of the position (*i.e.*, EC_{50}) and shape

Table 6. Quantitative aspects of the dose-response of key events in the uterotrophic response.

Key event	Study	Hill model parameters	Transition dose values	
			Starting points and slope-based TDVs (Murrell et al., 1998)	BMD$_{21}$ as a transitional dose (Sand et al., 2006)
Binding to ERa	Levin et al. (1993) (cytosol) (fractional binding response)	$K_d = 31.2$ nM Log $K_d = 1.49$ $n = 0.76$	(13.8, 102.8) (5.8, 62.4) **1.26 nM**	**5.41 nM**
	Levin et al. (1993) (nucleus) (fractional binding response	$K_d = 2.04$ nM Log $K_d = 0.310$ $n = 1.94$	(1.49, 27.3) (2.85, 51.8) **1.05 nM**	**1.03 nM**
	Notides et al. (1981)[a] (fractional binding response)	$K_d = 3.25$ nM Log $K_d = 0.512$ $n = 1.61$	(1.853, 7.48) (4.99, 20.7) **1.11 nM**	**1.43 nM**
Gene expression changes in relation to uterine weight gain (Naciff et al., 2003)	Naciff et al. (2003) (fold increase in Ca binding protein)	Bmax = 22.82 fold $K_d = 0.807$ µg/kg/d Log $K_d = -0.0930$ $n = 0.755$	(0.1, 3.5) (1.0, 12.5) **0.082 µg/kg/d**	**0.140 µg/kg/d**
	Naciff et al. (2003) (fold increase in uterine weight)	Bmax = 5.48 fold $K_d = 1.26$ µg/kg/d Log $K_d = 0.010$ $n = 0.914$	(0.1, 1.12) (1.0, 4.63) **0.171 µg/kg/d**	**0.240 µg/kg/d**
Gene expression changes in relation to uterine weight gain (Heneweer et al., 2007)	Heneweer et al. (2007) (fold increase in Ca binding protein)	Bmax = 12.66 fold $K_d = 5.35$ µg/kg/d Log $K_d = 0.728$ $n = 1.54$	(0.3, 2.62) (1.0, 9.1) **0.290 µg/kg/d**	**2.26 µg/kg/d**
	Heneweer et al. (2007) (fold increase)	Bmax = 3.8 fold $K_d = 15.65$ µg/kg/d Log $K_d = 1.195$ $n = 1.191$	(1.0, 2.02) (10.0, 3.79) **0.220 µg/kg/d**	**5.15 µg/kg/d**
Cell proliferation	Kaye et al. (1971) (increase in mitotic index	Bmax = 276 figures $K_d = 0.809$ µg/kg Log $K_d = -0.092$ $n = 2.21$	(1.5, 166) (15, 227) **0.0073 µg/animal/d**	**0.444 µg/animal/d**
Increase in blood flow measure by uterine peroxidase	Lyttle and DeSombre (1977)	Bmax = 69 units/g $K_d = 17.3$ µg/animal Log $K_d = 1.24$ N = 0.561	(1.0, 13.8) (10.0, 37.6) **0.053 µg/animal/d**	**1.64 µg/animal/d**
Uterine weight gain	Branham et al. (1985) (mg wet weight)	Bmax = 5.4 fold $K_d = 1.85$ µg/animal/d Log $K_d = 0.268$ $n = 0.271$	(0.1, 1.73) (10.0, 5.31) **0.078 µg/animal/d**	**0.014 µg/animal/d**

Here, the results of two methods for determining transitional dose values (TDVs) are shown. Details of the calculation methods are provided in the text. The form of the Hill model used here is shown in Table 7.

[a]Notides et al. (1981) observe the Hill coefficient for E2 binding to cell-free preparations ERα varies with the concentration of the receptor (from $n = 1.1$ at 0.3 nM ERα to 1.6 at 3.0 and 4.8 nM ERα, indicating that the Hill coefficient increases with increasing concentrations of ERα.

(*e.g.*, first- or second-order Hill plot) of the curve is possible. This level of information is needed for all events being considered as KEs. These data would be invaluable in eliminating proposed KEs for which the parameters of the dose-response curve are not compatible with those of the apical response. For example, a proximal event that displays a second-order Hill dose-response curve could not be a step in an apical response that exhibits a first-order Hill dose-response curve (Ong et al. 2010, Chow et al. 2011). In this way, quantitative dose-response modeling may provide some mechanistic insights into the role of various events (Simons and Chow 2012). In addition, various analytical tools can be employed to gain mechanistic insight that is available only when the Hill coefficient is equal to one (Dougherty et al. 2012, Ong et al. 2010). A Hill coefficient of two or greater may indicate involvement of transcription factors that act as dimers or higher-order multimers. Alternatively, the observation of Hill coefficients greater than one may also result from ligand-induced conformational changes in binding proteins that function as dimers or multimers

ers (Koshland 1996, Koshland and Hamadani 2002, Levitzki and Koshland 1969). Furthermore, it would be instructive to know the details of ligand binding to ERα in cell-free extracts, in whole cells and in whole animals. One would also want data on the genomic responses *in vitro* and in whole animals. In addition, these data would need to be of sufficient quality to support quantitative dose-response modeling.

Second, additional data are needed to provide dose-response information at different times for those events hypothesized to be KEs. Ideally, these data would be collected under the same experimental conditions as that for the apical event. When performed, interim sacrifices in a cancer bioassay often provide this type of data (*e.g.*, NTP 2006). Such data are necessary for constructing a time line of the KEs and providing data for the Dose-Time Concordance Table (Table 2).

Third, a decision should be made concerning the best experimental system for examining the effects of modulatory factors. For example, if ER-knockout mice are to be used, then high-quality dose-response data, as discussed above, should be collected from
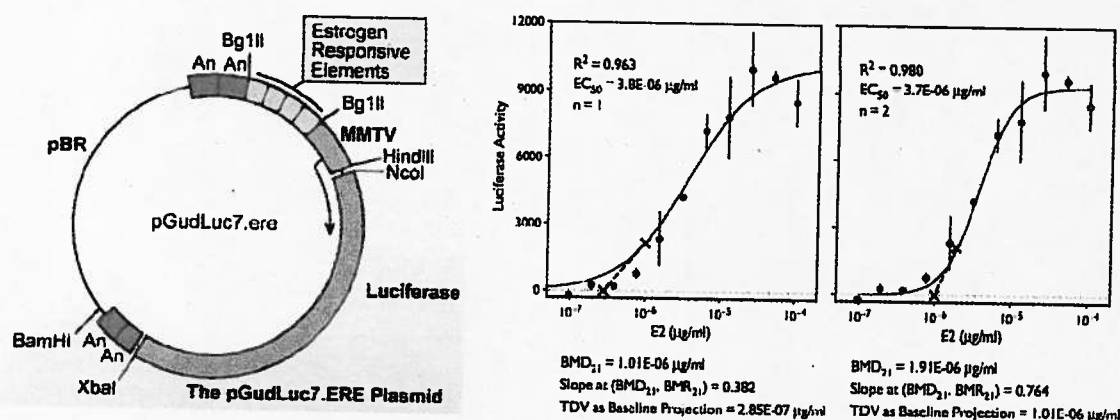
Figure 9. Details of one of the heterologous expression systems that could be used to substitute for the uterotrophic assay. Left: Stably transfected Luc reporter plasmid BG1Luc4E2 cell line from ICCVAM. Right: Concentration-response of the BG1Luc4E2 cells to estradiol showing fits to both first- and second-order Hill functions and the results of the transitional dose value calculation using the baseline projection method (Eq. 3,4 and 5). Please see Supplementary Content for another example.

both normal and knock-out mice. Alternatively, if tissue culture and high throughput studies are selected, then appropriate tissue culture lines could be used and would need to be identified.

Potential utility of understanding the MOA for uterotrophy. One potential result of the greater understanding deriving from more complete experimental data would be the potential for increased usage of in vitro assays measuring KEs and AEs as a screen to identify the chemicals to be assessed further in the uterotrophic assay, a scheme that is consistent with Tox21. The Q-KEDRF seems the best means of demonstrating this consistency. The Interagency Coordinating Committee on the Validation of Alternative Methods has validated a whole cell assay system (Figure 9; BG1Luc ER TA) to assess the activity of different test compounds. Yamasaki et al. (2002, 2003, 2004) measured the response of a reporter gene system as well as the uterotrophic response in whole animals but did not attempt to conduct IVIVE to determine the quantitative relationship between the two—both the reporter gene assay and the in vivo assay were used only for identification of biological effects.

One important aspect of uterotrophy as a model system is that it exemplifies the likely existence of thresholds in MOAs that include receptor binding as a KE. A TDV or range is located at the point where the rising portion of the dose-response begins (Murrell et al. 1998, Sand et al. 2006). Because the binding assays were conducted *in vitro* and the units of dose and routes of exposure were not consistent among the *in vivo* studies, it is difficult to draw conclusions about the numerical values of these either possible threshold values or TDVs, but the ability to estimate these values can, in some cases, provide great insight about the MOA (*e.g.*, Simon et al. 2009).

The value of the Hill coefficient can be important in determining whether linear or nonlinear extrapolation should be used for modeling various KEs or the Adverse Outcome. For the example of uterotrophy here, the ability to obtain insights from quantitative data is mitigated by the relative paucity of the data. Inspection of Figure 9 suggests that for this *in vitro* response in BG1LucE42 cells, both first and second order Hill models provide equally good fits to these data. Notides et al. (1981) did observe a shift in the Hill coefficient with increasing concentrations of ERα and attributed this increase to the

formation of homodimers with greater availability of ERα. The uterotrophic response itself is generally considered to follow a first-order Hill function but the data from Naciff et al. (2003) seem clearly second order, possibly for this reason. Additional data collection should provide greater certainty regarding the order of the Hill function.

Potential TDVs for the responses in Figures 8 were estimated using the baseline projection method of Murrell et al. (1998) and as the BMD_{21} value as noted by Sand et al. (2006; Table 6). Silkworth et al. (2005) also suggest a method for baseline projection. Details of this method are provided in the next section and in the Supplementary Content.

Alternative Dose Levels from the Hill function for ordering KEs. The Hill model is a three or four parameter equation for a nonlinear relationship between dose and response. The model was first applied by A.V. Hill in 1910 to describe the relationship between oxygen tension and saturation of hemoglobin (Hill 1910). In pharmacology and toxicology, the Hill model has been used extensively to describe the relationship between the dose of a xenobiotic and a biological response (Goutelle et al. 2008, Wagner 1968). In another very recent paper examining the shape and steepness of dose-response relationships for continuous endpoints, the Hill model and the exponential model were both found to provide adequate fits to a large number of data sets covering many continuous endpoints (Slob and Setzer 2014).

For consideration of MOA, location and steepness of the dose-response may help order the events within the dose range. One would wish to know the approximate dose at which the rising portion of the dose-response begins, in other words, the TDV.

A form of the Hill model is shown below and it will be used later to examine responses to estrogenic chemicals. We also provide in Table 7 the inverse equation for calculating the dose at a specified response, for example, the BMD, and the equation for the slope.

$$Response = g + \frac{(V_{max} - g)}{1 + 10^{n(\log_{10}(K_d) - \log_{10}(dose))}} \quad (2)$$

where g = background response;
Vmax = maximal response or efficacy;
n = Hill coefficient (unitless); and

Table 7. Inverse equations and slope equations of dose-response models from EPA's benchmark dose software (USEPA 2012) to enable estimation of baseline projection values.

Model	Equation	Inverse	Derivative
Hill	$Response = \dfrac{1}{1+10^{n(\log_{10}(K_d)-\log_{10}(dose))}}$	$\log_{10}(dose) = \log_{10}(K_d) - \dfrac{\log_{10}\left(\dfrac{1}{Response}-1\right)}{n}$	$Slope = \dfrac{n10^{n((\log_{10}(K_d)-\log_{10}(dose))}}{dose\left(10^{n((\log_{10}(K_d)-\log_{10}(dose))}+1\right)^2}$
Logistic	$Response = \dfrac{1}{1+e^{-(\alpha+\beta dose)}}$	$dose = \dfrac{-\alpha - \log_e\left(\dfrac{1}{Response}-1\right)}{\beta}$	$Slope = \dfrac{\beta e^{-(\alpha+\beta dose)}}{\left(1+e^{-(\alpha+\beta dose)}\right)^2}$
Log-Logistic or Dichotomous Hill	$Response = \dfrac{1}{1+e^{-(\alpha+\beta \log_e(dose))}}$	$\log_e(dose) = \dfrac{-\alpha - \log_e\left(\dfrac{1}{Response}-1\right)}{\beta}$	$Slope = \dfrac{\beta e^{-(\alpha+\beta \log_e(dose))}}{dose\left(1+e^{-(\alpha+\beta \log_e(dose))}\right)}$
Multistage (2nd order)	$Response = 1-e^{-\beta_1 dose-\beta_2 dose^2}$	$dose = \dfrac{-\beta_1 + \sqrt{\beta_1^2 - 4\beta_2 \log_e(1-Response)}}{2\beta_2}$	$Slope = e^{-\beta_1 dose-\beta_2 dose^2}(\beta_1 + 2\beta_2 dose)$
Weibull	$Response = 1-e^{-\beta dose^\alpha}$	$\log_e(dose) = \dfrac{\log_e\left(\dfrac{-\log_e(1-Response)}{\beta}\right)}{\alpha}$	$Slope = \alpha\beta \, dose^{\alpha-1}e^{-\beta dose^\alpha}$
Exponential Model 2	$Response = \alpha e^{\beta dose}$	$dose = \dfrac{\log_e\left(\dfrac{Response}{\alpha}\right)}{\beta}$	$Slope = \alpha\beta e^{\beta dose}$
Exponential Model 3	$Response = \alpha e^{\beta dose^\delta}$	$\log_e(dose) = \log_e\left(\dfrac{\log_e\left(\dfrac{Response}{\alpha}\right)}{\beta}\right)\left(\dfrac{1}{\delta}\right)$	$Slope = \alpha\beta e^{\beta dose^{\delta-1}}e^{\beta dose^\delta}$

These values may be useful for ordering events within a hypothesized MOA. These equations are written to be easy to implement in spreadsheet software such as MS-Excel. Their use is not for development of regulatory criteria but rather exploration of hypothesized MOAs.

K_d = affinity or dose at the half-maximal response, a measure of potency (For concentrations, this parameter is often shown as EC_{50}, indicating a dose or concentration with a 50% of maximal efficacy.

In Eq. (2) and all equations following, common or base 10 logarithms are denoted by "$\log_{10}$" and natural logarithms are denoted by "$\log_e$." All the responses shown in Figure 8 were fit to Eq. (2). The third column in Table 6 shows the fitted values for K_d and n, the Hill coefficient.

Another method to obtain the TDV is that of Murrell et al. (1998). The baseline projection of the rising part of the curve is obtained by choosing two points by inspection, one above and one below the half-maximal response. The slope of the rising portion is calculated as the ratio of the differences of the dose and response values of these two points.

$$Slope = \frac{R_1 - R_2}{\log_{10}(dose_1) - \log_{10}(dose_2)} \qquad (3)$$

where R_i = fractional response levels above and below 0.5.

This slope will likely be very close to that at the half-maximal response. Hence, using 0.5 as the measure of the response at the K_d value on a zero-to-one scale, the dose at the onset of the rising portion of the dose-response is calculated as:

$$TDV = \log_{10}(K_d) - \frac{0.5}{Slope} \qquad (4)$$

The results are shown in column 4 of Table 6.

For the form of the Hill model shown in Eq. (2), the dose at any fractional response level, for example, 0–1, can be obtained as follows:

$$\log_{10}(dose) = \log_{10}(K_d) - \frac{\log_{10}\left(\dfrac{1}{Response}-1\right)}{n} \qquad (5)$$

Equation (5) was used to calculate the BMD_{21}, identified as a TDV by Sand et al. (2006; Table 6).

Once the Hill model parameters for the dose-response (Eq. 2) have been obtained from fitting software, the results of Eqs. (3–5) can be easily obtained with spreadsheet software or even a hand calculator. Only the Hill coefficient, n, and the common logarithm of the half-maximal concentration, $\log_{10}(K_d)$, are needed.

These doses are referred to as transitional because their location marks the approximate transition to the rising portion of the dose-response (Sand et al. 2006). The method of Murrell et al. (1998) explicitly considers steepness with a calculation of the slope. The BMD_{21} is the point at which the generalized Hill model transitions to the rising phase, as indicated by higher derivatives of the model (Sand et al. 2006).

Measurements of binding to the estrogen receptor show very similar slope-based TDVs. One might expect gene expression changes to occur at a lower dose than uterine weight gain. The slope-based TDV for the increase in expression of vitamin D-dependent intestinal calcium-binding protein (Calb3) from Naciff et al. (2003) is about half than that for uterine weight gain in the same study; however, the BMD_{21} values for these two effects are much more similar (Table 6).

In contrast, the data from Heneweer et al. (2007) show about a two-fold increase in the BMD_{21} but similar slope-based TDVs. Both studies used immature female Sprague-Dawley rats so the difference in the relationship of TDVs between the two studies is likely due to the small number of data points and uncertainty in the fit. The fact that these two methods of calculating a transitional dose range/value give different results for two similar studies would be a reason to obtain further details of the biological role of Calb3 in the uterotrophic response. Highlighting the need for additional qualitative information about the biology underlying the MOA is a great benefit of the use of the Q-KEDRF.

Confidence limits could be likely determined for these TDVs, but the point of their use is to obtain evidence regarding the timing and role of events in a hypothesized MOA. The relationship between Calb3 and uterine weight is not yet known (Naciff et al. 2003, Heneweer et al. 2007). Hence, a review of the literature and possibly some laboratory studies would go further in addressing this particular data gap.

Last in the table are three measurements for increases in uterine cell proliferation, blood flow, and weight gain reported in OECD (2003). All three studies were conducted in rats and the TDVs may suggest that the order of events along the dose continuum is:

1) cell proliferation;
2) increased blood flow measured by uterine peroxidase; and,
3) uterine weight gain.

Both types of TDV for all three studies were expressed in units of µg/animal/d. Here, the slope-based TDV suggests that cell proliferation may be a low dose–response, whereas the slope-based TDVs for increases in blood flow and uterine weight gain occur fairly close to each other along the dose continuum. The TDVs as the BMD_{21} for these three responses are more challenging to interpret. The reason is likely that the slope-based TDVs used the actual data to develop a slope value and the BMD_{21} TDV uses the fitted Hill coefficient. In all three cases, the fitted Hill coefficients had low values and the fits were performed on data with six or fewer dose values.

Another example of this type of quantitative MOA analysis can be found in recent work on the MOA of dioxin liver carcinogenesis in rats (Budinsky et al. 2014, Simon et al. 2009). Both papers present figures showing dose-response plots of different events in the MOA ordered by increasing K_d values and increasing Hill coefficients.

In all likelihood, statisticians can think of much more sophisticated analyses using the slope of the dose-response. Such approaches could use expressions for the slope of the dose-response and attempt to discover in what dose ranges the most rapid change occurs. However, for the purposes of working out events within a hypothesized MOA, easily calculated values such as K_d or the TDV can be very useful.

There may be additional insight gained from using a baseline projection method similar to that obtained at the half-maximal response level using the procedure of Murrell et al. (1998). Table 7 provides equations for commonly used empirical dose-response models, the corresponding inverse equations that solve for dose as the independent variable based on a chosen response, and equations for the dose-response slope at any point. In some instances, these equations can be used to project to the baseline or zero response using the slope at the chosen level of response (Figure 9; Supplementary Content). The inverse equations in Table 7 simply express the dose corresponding to a chosen fractional response (assuming "1" is the maximal response). Using these equations should prove simpler than obtaining an implicit solution. The slope equations in Table 7 provide a means of calculating the slope at the benchmark point (BMD, BMR).

Baseline projection from the 21% response level is shown graphically in Figure 9. Although the values for the EC_{50} are very close, the BMD_{21} values differ by a factor of 2 and the baseline projections from the 21% response level differ by over three-fold. An examination of these differences may help discover the sequence of KEs in a proposed MOA.

As noted, the Supplementary Content provides another example calculation of this baseline projection method that incorporates both the location and steepness of the dose-response at a chosen point and how to use such information in thinking about a hypothesized MOA.

Comparing the values of the Hill coefficients of various events in a hypothesized MOA may provide additional insight and contribute to the decision of whether to assume the adverse outcome follows a linear or nonlinear MOA. Ligand binding and the constellation of early steps in gene transcription may have Hill coefficients close to unity and thus their dose-response might be considered linear (Murrell et al. 1998, Budinsky et al. 2014). KEs that have Hill coefficients with values of 2 or greater invariably indicate the MOA for the adverse outcome will be nonlinear (Chow et al. 2011).

Log-steepness, measured by the ratio of the BMD_{10} to the BMD_{05}, was considered for use in ordering events with a hypothesized MOA (Slob and Setzer 2014). The dose-response data provided in EPA (2005c) was used to obtain values of log-steepness for KEs in the MOA of cacodylic acid (Tables 2 and 3; Figure 3). The three KEs are cytotoxicity, proliferation, and hyperplasia occurring at 10 weeks (Table 3). Appendix D of this EPA publication contains the BMDS output for these three KEs. The values for log-steepness calculated as the BMD ratio for these three KEs (cytotoxicity, proliferation, and hyperplasia) were 2.1, 1.1, and 1.4, respectively. Slob and Setzer (2014) note that log-steepness estimated as the BMD ratio is imprecise, and, while this is only a single example, this easily calculated value did not prove helpful in ordering KEs within a hypothesized MOA. Further work is needed to determine whether this measure of log-steepness can indeed help inform details of MOA.

Constructing a Dose-Time Concordance Table may also help to identify late occurring KEs. These late KEs in the modes of action of complex adverse outcomes such as cancer or developmental effects, may be highly nonlinear and will likely have high-valued Hill coefficients (Brown et al. 2012, Hanahan and Weinberg 2000, 2011, Simon et al.

2009). In some cases, sufficient information about the MOA will be available to select some KEs to use as appropriate precursors to the adverse outcome such as was done by EPA for dimethylarsinic acid. The ability to select appropriate precursor KEs will require quantitative knowledge of the relationship between that KE and the adverse outcome. When the knowledge is available, such precursor events can be used as the basis for risk assessment (Simon et al. 2009, USEPA 2005a, Thompson et al. 2014).

Application of knowledge of the MOA for uterotrophy in risk assessment. A number of host, life stage, and environmental factors likely will modulate human responses to chemicals shown to be estrogenic in the uterotrophic assay and in surrogate in vitro assays. Because many potentially estrogenic chemicals contain one or more hydroxyl groups that interact with specific ligand-binding pockets in ERα, the metabolism of these chemicals in the enterocytes lining the gastrointestinal tract and the liver may result in their inactivation. Hence, for some chemicals, first pass serves as a detoxification process.

For example, bisphenol A (BPA) is almost completely inactivated by phase II metabolism in enterocytes and liver by both glucuronidation and sulfation. These processes occur in both humans and rats (Hengstler et al. 2011). Differences in glucuronidation and sulfation of BPA in rats and humans exist and may provide the basis for interspecies extrapolation of metabolism and consequent bioavailability of BPA (Mazur et al. 2010). Alternatively, these data may be used to improve PBPK models of BPA (Fisher et al. 2011, Teeguarden et al. 2005).

Modulating factors for estrogenic responses in humans. After oral ingestion, it is not possible to detect free BPA in plasma in adult humans (Willhite et al. 2008). PBPK modeling suggests that levels of free BPA in very young children may be higher than in adults due to lower glucuronidation capacity during the first 2 months of life (Edginton and Ritter 2009, Mielke and Gundert-Remy 2009). Free BPA has been detected in the urine of premature infants in neonatal intensive care and its source may be medical devices and the need to deliver medical interventions directly via the blood (Calafat et al. 2009). In contrast, free BPA has not been detected in the urine of full-term healthy infants up to 44 days in age (Nachman et al. 2013). This fact suggests that the glucuronidation capacity in healthy infants is sufficient to metabolize BPA from environmental exposures.

Polymorphisms in uridine 5'-diphospho-glucuronosyltransferase enzymes that conjugate glucuronide may be a potential ModF (Allegaert et al. 2009, Court 2010, Girard et al. 2007, Guillemette et al. 2010, Krekels et al. 2012, Mercke Odeberg et al. 2006, Miyagi and Collier 2011, Strassburg et al. 1997, de Wildt et al. 1999). As noted, differences in glucuronidation occur with gender and age. Diet may also be a factor in the ability to inactivate estrogenic chemicals (Navarro et al. 2009, 2011, Saracino et al. 2009). In all cases of oral exposure, the actual exposure needs to be considered in a quantitative fashion—the inability to detect free BPA in the urine of normal infants suggests that exposures may be sufficiently low that glucuronidation is essentially complete (*e.g.*, Ye et al. 2012). There may be exposures to estrogenic chemicals by routes other than oral, for example, dermal or inhalation, for which glucuronidation does not occur. However, these exposures appear to be miniscule (Geens et al. 2012).

The occurrence of male reproductive tract pathologies in offspring of women administered diethylstilbestrol (DES) during pregnancy suggests that both a lowest-observed-adverse-effect level and a NOAEL exist for these developmental effects. Because no formal clinical trials had been conducted with DES, the total dose varied among clinics by an order of magnitude or more. Male reproductive tract abnormalities were observed in offspring of mothers receiving higher total doses of DES, that is, 12–18 g during pregnancy (Dietrich 2010, Golden et al. 1998), whereas no clear increase was observed in reproductive tract effects in offspring of mothers administered 1.4 g of DES during pregnancy (Leary et al. 1984).

Exposure to more than one estrogenic chemical, such as dietary phytoestrogens, may interact with, or complement, endogenous or other exogenous chemicals. As noted, at sufficient doses, estrogenic chemicals act as anti-androgens in males. However, dose addition of these chemicals is unlikely unless at least two of the doses occur in the rising portion of the dose–response curve (Borgert et al. 2012). Quantitative aspects of dose-response such as affinity, efficacy, and potency need to be considered for chemicals that act via receptor binding—simply using dose addition and some measure of relative potency will be inadequate for risk assessment (Borgert et al. 2012).

The examination of the MOA for uterotrophy requires *in vivo* measurement of the adverse outcome/apical endpoint and includes *in vitro* measurements of the MIE, genomic data, and physiological measures of KEs. Hence, this example demonstrates the use of data from tiers 1–4 of the toxicity resource pyramid of the RISK21 Roadmap (Figure 1), and illustrates the strength of MOA analyses in terms of generating data useful for risk assessment purposes.

Discussion

The MOA/HRF along with the Q-KEDRF described here provides a strong foundation for using the information gathered as a means of reducing uncertainty in risk assessments. The KEDRF laid out the approach for harnessing the extensive available data for the KEs within a putative MOA. The Q-KEDRF provides additional tools with which to gain further insights about how the KEs relate to each other and to the adverse outcome/apical event in a quantitative way in both the dose- and time-dimensions.

In risk assessment, the greatest quantitative impact comes from the choice of a linear approach versus a nonlinear approach for modeling the dose-response for the critical effect or apical effect of concern. The dose-responses for the KEs can be used to inform the shape of the dose-response for the apical effect of concern. For receptor-mediated effects, as noted, quantitative dose-response modeling can provide much greater understanding. For example, if the dose-responses of some or all KEs exhibit biological thresholds, for example, cytotoxicity of the liver and kidney induced by chloroform (Andersen et al. 2000), then the combination of events will also display a dose threshold. Alternatively, if the dose-responses for KEs do not exhibit dose thresholds, then the combination of events may result in a linear dose-response for the apical event. The ability to calculate possible threshold or transition dose values from quantitative dose-response modeling provides a means to determine whether linear or nonlinear extrapolation is appropriate (Table 7).

It is increasingly clear that account has to be taken of those ModFs that could influence the shape of the dose–response curve, the efficacy or magnitude of the apical response or selected critical effect, and the potency or location along the dose continuum. For example, how much variation can be expected for a particular ModF? Again, this depends on the underlying biology. Sufficient variation may "linearize" the dose-response of the apical event (Conolly et al. 2005, Lutz 2001). The question then is: will this amount of variation "linearize" the population dose-response to a sufficient extent to support the choice of linear low-dose extrapolation? As a generalization, ModFs that are likely to modify the dose-response characterization as part of the risk assessment process will be relatively frequent in the population (given that dose-response is a population feature). Some of these ModFs are "inevitable" and are characteristics of the general population (sex, age, and genotype); others are "manageable" and are characteristic of specific subpopulations (smoking, diet, and weight). Additional research on this topic and the overall role of ModFs is essential to inform the consideration of ModFs and their effect on MOA as part of problem formulation.

At this point in the history of risk assessment, the utility of the Q-KEDRF remains to be determined: experience in conducting real-world risk assessments will demonstrate any value added. Certainly, the Dose-Time Concordance table and Dose-Response Species Concordance table for KEs and ModFs (Tables 2–4) should provide a significant amount of help. The National Research Council recently reviewed EPA's Formaldehyde risk assessment and as part of that review, suggested that the documentation for chemical-specific risk assessments in the IRIS program be organized around informative tables (NRC 2011). The Dose-Time and Dose-Response Species Concordance tables could be very useful in that effort.

At present, the full utility of the Q-KEDRF has barely begun to be realized. The example of rat uterotrophy, while being arguably the best documented physiological response to the extensively studied steroid hormones, clearly demonstrates not only the shortcomings in the available data but also how much actual insight can be acquired through the development of a Q-KEDRF for a specific response. The Q-KEDRF will likely change as experience in using it is gained. Nonetheless, some of the basic issues discussed here will likely become hallmarks of any framework implemented to understand the MOA of a particular adverse outcome. These issues include: (1) separating KEs from putative KEs and (2) understanding the relationship between KEs based upon their dose-response and the timing of their occurrence. Use of this information can significantly improve risk assessments by reducing uncertainty and fostering the incorporation of this information into easy-to-use tables.

Acknowledgments

Declaration of interest

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